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MULTIVALENT FORMATION AND GENETIC SEGREGATION IN SOME ALLOPOLYPLOID GOSSYPIUM HYBRIDS[1]

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THE relationships between segregation and cytology have been investigated in only a few polyploid hybrids. A study by SKALIŃSKA in 1935 on the segregation of parental characters in allopolyploid Aquilegia showed that flower color segregated while other characters did not. This segregation was assumed to result from heterogenetic pairing of chromosomes since about one to two quadrivalents per cell were observed. Segregation in an allopolyploid of *Bromus ciliatus* × *B. laevipes* also reflected a high amount of multivalent pairing (JAKOB 1952). However, segregation ratios may not always depend on multivalent formation since DAWSON (1941) observed almost exclusively bivalent associations in *Lotus corniculatus*, yet obtained tetrasomic 5:1 ratios for the inheritance of cyanogenesis. In allopolyploids of *Elymus glaucus* × *Sitanion spp.*, STEBBINS and VAARAMA (1954) found only a small amount of segregation for parental characters that differentiated the species, yet the mean number of multivalents ranged from four to five multivalents per sporocyte. In explanation, the genome formula was assumed to be $A_2A_2A_2A_2E_1E_1E_8E_8$ with the multivalents involving chiefly the A_2 genomes; the small amount of segregation was ascribed to rarity of pairing of the E genomes which carried the factors distinguishing the species. A correlation of cytological behavior with genetic segregation was attempted by FAGERLIND (1937) in allotetraploid *Galium Mollugo* × *verum*. He established certain formulae which related inter- and intraspecific bivalent and multivalent syndesis to the amount of genetic segregation in selfed allopolyploids.

In cotton breeding work it is often desirable to transfer certain genes from wild species to cultivated cottons by means of interspecific crosses. The problem, however, is not simple since the cultivated New World cottons (n=26) are allopolyploids while the wild cottons are diploids (n=13). In synthetic amphiploids from these species the amount of segregation should be related to the extent of homogenetic and heterogenetic pairing (WADDINGTON 1939). If pairing is completely homogenetic, only bivalents are formed from homologues and there would be no segregation of genes differentiating the parental species; consequently, transfer of genes would not be possible. However, if pairing is heterogenetic, segregation

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would result from both bivalents and multivalents. The results of a comparative study of segregation and multivalent formation are, therefore, of both theoretical and practical interest.

Segregation studies in various hexaploid and tetraploid hybrids were made in this laboratory by GERSTEL (1956) and GERSTEL and PHILLIPS (1957). New World cottons contain two genomes designated A and D (BEASLEY 1942) while the wild cottons selected had either the A, B, or D genomes. Tetrasomic ratios were obtained in 4n (*G. arboreum* × *herbaceum*) ($A_1A_1A_2A_2$) for pollen color, plant anthocyanin, leaf shape, and "red lethal". In contrast, only rare segregants (mean backcross ratio for several loci 76.5:1) were obtained in Old World × *anomalum* tetraploids (AABB). (Old World designates *G. arboreum* and *G. herbaceum*). In hexaploid *G. hirsutum* × *raimondii*, ($AADDD_2D_2$), segregation for the D genome character "*RAI*" spot was 8.68:1. The hexaploid of *G. hirsutum* and *G. thurberi*, ($AADDD_1D_1$), in contrast, gave a wider ratio (47.2:1) for two D loci.

The cytology of these synthetic amphiploids had previously been studied by several workers (Table 1). Amphiploids from different strains of *G. herbaceum* and *G. arboreum* showed rather high numbers of multivalents (IYENGAR 1944a). For 4n (*G. arboreum* × *anomalum*) and 6n (*G. hirsutum* × *anomalum*) much fewer multivalents were reported (IYENGER 1942, 1944b). In the hexaploid *G. barbadense* × *thurberi*, ($AADDD_1D_1$), IYENGAR (1944b) observed only 2.46 multivalents per cell while in the hexaploids of *G. hirsutum* × *thurberi* and *G. hirsutum* × *raimondii*, BROWN and MENZEL (1952) saw 5.03 and 4.65 multivalents, respectively. Since multivalent formation in the last two hexaploids was similar, according to the latter authors, while the amount of segregation differed considerably, according to GERSTEL (1956), it was felt desirable to re-examine the cytology of the various tetraploids and hexaploids using the same plants for which segregation data were available. Other factors such as chiasma frequency and environmental effects which might have contributed to the discrepancies between the segregation ratios and the cytology were also considered.

MATERIALS AND METHODS

The hexaploids and tetraploids used in this study were synthesized from various wild and cultivated Gossypium species. Detailed descriptions of the stocks of *G. hirsutum*, *G. barbadense*, etc., and the methods used to synthesize the allopolyploids have been described by GERSTEL (1956). Cytological studies were made on these same plants in the colchicine treated generation. The following hexaploids were studied:

6n(*G. hirsutum* S4025 × *thurberi*)
6n(*G. hirsutum* S4025 × *raimondii*)
6n(*G. hirsutum* S5082 × *raimondii*)
6n(*G. hirsutum* SM4 × *raimondii*)
6n(*G. barbadense* TZVR × *raimondii*)

TABLE 1

Chromosome associations of various allopolyploids as reported in the literature

Allopolyploid	Mean frequencies of I	II	III	IV	V	VI	No. PMC's	Author reporting
3n(*G. thurberi* × *hirsutum*)	13.06	12.70	0.18	...	...	...	50	WEBBER 1939
3n(*G. hirsutum* × *thurberi*)	13.5	12.45	0.2	...	...	...	20	SKOVSTED 1937
3n(*G. hirsutum taitense* × *thurberi*)	13.4	12.05	0.5	...	...	...	20	SKOVSTED 1937
3n(*G. hirsutum* × *raimondii*)	12.57	11.65	0.87	0.125	...	...	40	BOZA-BARDUCCI, MADOO 1941
4n(*G. herbaceum* × *arboreum*)	1.03	10.50	0.30	7.27	...	...	30	IYENGAR 1944a
4n(*G. herbaceum* × *arboreum*)	0.98	12.59	0.53	6.08	...	...	49	IYENGAR 1944a
4n(*G. herbaceum* × *arboreum*)	1.45	11.55	0.28	6.66	...	...	29	IYENGAR 1944a
4n(*G. arboreum* × *anomalum*)	0.40	22.67	0.42	1.25	...	...	..	IYENGAR 1942
6n(*G. barbadense* × *thurberi*)	0.67	33.96	0.42	2.00	0.00	0.04	24	IYENGAR 1944b
6n(*G. hirsutum* × *thurberi*)	0.90	28.72	0.59	4.40	0.04	...	22	BROWN and MENZEL 1952
6n(*G. hirsutum* × *raimondii*)	0.93	27.93	0.31	4.34	...	...	29	BROWN and MENZEL 1952
6n(*G. hirsutum* × *anomalum*)	2.90	35.43	0.50	0.60	0.07	...	30	Corrected from IYENGAR 1944b

The first two types were studied under greenhouse conditions during the winter seasons of 1953–54 and 1954–55 and the remaining ones only in the latter year. In order to have more cytological material, branches of two sibs from both 6n(*G. hirsutum* S4025 × *thurberi*) and 6n(*G. hirsutum* S5082 × *raimondii*) were grafted onto a commercial cotton thus multiplying the number of available plants. Two sib plants were available of 6n(*G. barbadense* × *raimondii*), three of 6n(*G. hirsutum* SM4 × *raimondii*), and five of 6n(*G. hirsutum* S4025 × *raimondii*).

The tetraploids analyzed are listed below:

4n(*G. herbaceum* × *anomalum*)
4n(*G. arboreum* "Sanguineum" × *anomalum*)
4n(*G. arboreum* S5133 × *anomalum*)
4n(*G. arboreum* "Sanguineum" × *herbaceum*)
4n(*G. thurberi* × *raimondii*)

The first four tetraploids were raised in the field in the summer of 1953. Two sibs of 4n(*G. arboreum* "Sanguineum" × *anomalum*) were grown; the other hybrids were represented by only one plant. Grafts from each of these plants were carried through the winter in the greenhouse and in the spring were set in replicate into the field and into containers. Sibs of 4n(*G. thurberi* × *raimondii*) were grown in the greenhouse in the winters of 1954–55 and 1955–56. To test the effects of temperature on multivalent and chiasma formation one plant of 4n(*G. arboreum* "Sanguineum" × *anomalum*) was placed in an air-conditioned room where the temperature varied from 23–27°C which was considerably below the maximum temperatures obtained outdoors in summer.

Buds were fixed in a mixture of one part chloroform, one part alcohol, one part acetic acid, supersaturated with mercuric chloride; other modifications of Carnoy's mixture were also used. The pollen mother cells (PMC's) were squashed in acetocarmine using one anther per slide. Spreading seemed to be improved by coating the cover slips with the commercial organo-silicon compound "Desicote" (Schultz and Hungerford 1953). Only cells in which all the chromosomes could be counted and the multivalents accurately determined were used in calculating the averages. In determining the types of associations, most of the cells were also checked by Dr. D. U. Gerstel. Multivalent averages were obtained by counting associations of 3, 4, 5, or 6 chromosomes as one multivalent, and those with 7 or 8 as two. Question marks after the values of the higher multivalents in the tables refer only to uncertainty as to the precise number of chromosomes in the multivalent associations and did not involve a question of the presence of a multivalent. The shapes of the multivalents were classified from drawings of those cells in which all multivalents could be analysed.

Since the number of chiasmata is difficult to determine with certainty in the small cotton chromosomes, chiasma counts were made using two different methods. First, the assumption was made that there was a maximum of one half chiasma per chromosome arm—thus bivalents would have a maximum of two chiasmata by this arbitrary *method a*. Secondly, since some bivalents definitely appeared to have more than one half chiasma per arm, the counts of *method b* were based on the actual appearance of the association.

Microcytes and pollen grains were also examined in acetocarmine. In the tetrad analysis one anther was used per slide, and counts were made only from intact tetrads. The number of microcytes in the hexaploids was determined monthly for about one year from three different positions in the bud: top, middle, and bottom. Pollen grains from the same locations were compared in the following hexaploids: *G. hirsutum* (S4025 and S5082) × *raimondii* and *G. hirsutum* S4025 × *thurberi*. They were classed according to color with only large bright red grains being considered good.

Ovules were counted using the same flowers as in the pollen studies. The number of ovules in each locule was counted separately, and fruits with three locules were kept apart from those with four locules. Seeds and motes (immature or aborted seeds) were counted in mature fruits of crosses of 6n(*G. hirsutum* S4025

× *thurberi*) and 6n(*G. hirsutum* S5082 × *raimondii*) with *G. hirsutum*.

Statistical tests of significance were made by means of chi-square and t-tests (SNEDECOR 1946).

RESULTS

Multivalent formation. The mean frequencies and ranges of the different associations observed in the various amphiploids are given in Table 2 and the average number of multivalents in Table 9. Whenever possible, tests of significance were made to see if the averages for the different years, stages, and buds could be combined. Nonsignificant differences were obtained between the values for two years in 6n(*G. hirsutum* S4025 × *thurberi*) and 6n(*G. hirsutum* S4025 × *raimondii*). In all the tetraploid hybrids, the data for the two years were also homogeneous. Diakinesis and metaphase I stages in 6n(*G. hirsutum* S5082 × *raimondii*) showed nonsignificant differences. Similarly, averages of three buds of

TABLE 2

Observed mean frequencies of chromosome associations per pollen mother cell

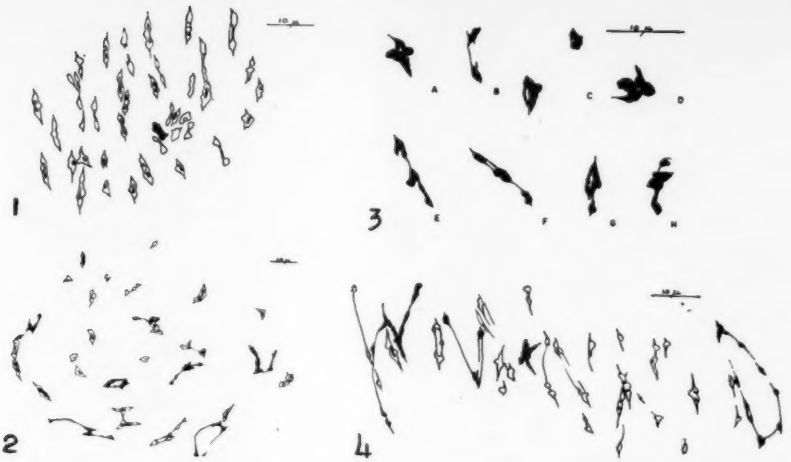
Amphiploid	No. of PMC's	Average number of I	II	III	IV	V	VI	VIII	Ranges of multivalents
6n(*G. hirsutum* S4025 × *thurberi*)	44	1.52	31.06	0.29	3.23	0.00	0.09?	...	0–9
6n(*G. hirsutum* S4025 × *raimondii*)	47	1.26	27.51	0.49	4.81	0.00	0.17?	...	2–11
6n(*G. hirsutum* S5082 × *raimondii*)	85	0.42	26.49	0.33	5.76	0.02	0.07	...	2–11
6n(*G. hirsutum* SM4 × *raimondii*)									
plants F and J	10	1.00	28.70	0.50	4.40	0.10?	...	...	2–8
plant D	11	0.82	26.73	0.64	5.18	0.00	0.18?	...	3–11
6n(*G. barbadense* TZVR × *raimondii*)									
4/7/55	12	1.00	31.92	0.33	2.92	0.00	0.08	...	1–8
4/22/55	6	1.33	25.83	0.67	5.50	0.00	0.16	...	
4n(*G. herbaceum* × *anomalum*)	78	0.78	22.00	0.13	1.54	0.01	0.10	...	0–5
4n(*G. arboreum* "Sanguineum" × *anomalum*)	61	0.70	21.31	0.26	1.65	0.05	0.15	0.02	0–5
cold treated (29 days)	14	0.21	21.21	0.14	1.93	0.07	0.14?	...	0–5
4n(*G. arboreum* S5133 × *anomalum*)	15	0.93	22.73	0.27	1.20	...	...	...	0–3
4n(*G. arboreum* × *herbaceum*)	12	0.33	6.83	0.17	8.50	0.00	0.25?	0.25	4–13
4n(*G. thurberi* × *raimondii*)	16	0.62	17.75	0.19	3.56	0.06	0.12	...	1–8

6n(*G. hirsutum* S5082 × *raimondii*) picked on the same day did not differ. However, from a *G. barbadense* × *raimondii* hexaploid two bud collections only two weeks apart were highly significantly different—in fact the data from the earlier collection were like those from 6n(*G. hirsutum* × *thurberi*) while the results of the later collection were like those from 6n(*G. hirsutum* S4025 and S5082 × *raimondii*). Because of this discrepancy, no average value was calculated for this amphiploid.

Another question of interest concerned the influence of cold temperatures upon multivalent formation. One bud from a 4n(*G. arboreum* "Sanguineum" × *anomalum*) plant which was subjected to cold for 29 days had the same range and almost the same average number of multivalents (2.28) as the field grown plants (2.25).

After the averages for multivalent frequencies of the various amphiploids were computed, comparisons were made between them (see Table 9 for these averages). As might be expected, the two hexaploids *G. hirsutum* (S4025 and S5082) × *raimondii* did not differ significantly and their means could be combined. In contrast, when the multivalent frequency distribution for the *G. hirsutum* × *thurberi* hexaploid was compared with the combined value of the two *G. hirsutum* × *raimondii* hexaploids, highly significant differences were obtained. Among the tetraploids, 4n(*G. herbaceum* × *anomalum*) and 4n(*G. arboreum* "Sanguineum" × *anomalum*) had similar ranges of multivalents and were homogeneous while 4n(*G. arboreum* S5133 × *anomalum*) had a slightly lower value. Although 4n (*G. arboreum* × *anomalum*) but not 4n(*G. herbaceum* × *anomalum*) is capable of forming a translocation ring of eight chromosomes (GERSTEL 1953), the multivalents from all three amphiploids involving *G. anomalum* were averaged. The mean frequency of multivalents in the amphiploid between the Old World species, 4n(*G. arboreum* "Sanguineum" × *herbaceum*), was significantly higher than any of the other amphiploids; however, only a few cells could be analyzed with confidence because of the many multivalents present. The mean frequency of multivalents in 4n(*G. thurberi* × *raimondii*) was highly significantly different from those of any other tetraploid and slightly, but not significantly, lower than that of 6n(*G. hirsutum* × *thurberi*).

Two of three 6n(*G. hirsutum* SM4 × *raimondii*) sib plants (F and J) were easily analyzed; but the third plant, D, in spite of having well spread PMC's, was more difficult. Some of the bivalents in plant D appeared to have a higher number of chiasmata per arm (Figure 3D), were abnormally shaped (Figure 3B), or were very small (Figure 3C, a typical bivalent was drawn for comparison). Abnormal trivalents (Figures 3E-G) and an unusual configuration which might have been a quadrivalent (Figure 3H), were also observed. Mean numbers of multivalents per PMC were 5.0 for F and J and 6.0 for D and were not significantly different from each other or from 6n(*G. hirsutum* S4025 and S5082 × *raimondii*). The data of plant D, because of these abnormalities, were kept separate from its sibs, and the data of plants F and J were not combined with the means of the other 6n(*G. hirsutum* × *raimondii*) stocks.



FIGURES 1.–4.—Metaphase configurations of Gossypium amphiploids. Multivalents shown solid; line represents 10μ in each figure (camera lucida). FIGURE 1.—6n(*G. hirsutum* S4025 × *thurberi*). 37 II + 1 IV. FIGURE 2.—6n(*G. hirsutum* S4025 × *raimondii*). 24 II + 6 IV + 1 VI? FIGURE 3.—Abnormal configurations in hexaploid Gossypium. A. Bivalent with probably four chiasmata. B-H. 6n(*G. hirsutum* SM4 × *raimondii*) plant D. B. Abnormal bivalent. C. Normal bivalent and very small bivalent from the same PMC. D. Bivalent or quadrivalent. E.-G. Abnormal trivalents. H. Quadrivalent? FIGURE 4.—4n(*G. thurberi* × *raimondii*). A PMC with 17 II + 3 IV + 1 VI and 41 chiasmata.

A typical cell of 6n(*G. hirsutum* S4025 × *thurberi*) with one quadrivalent is illustrated in Figure 1. An example of a cell of 6n(*G. hirsutum* S4025 × *raimondii*) with more multivalents (one possible VI and six IV's) is pictured in Figure 2. Six of the bivalents appeared to be connected by "sticky strands". Such sticky configurations were hard to analyze and were arbitrarily classed as bivalents. A metaphase configuration of tetraploid *G. thurberi* × *raimondii* is illustrated in Figure 4; the presence of a ring hexavalent is unusual and unexplained. Complete conjugation, 11 IV + 1 VIII, was observed in only one PMC (Figure 5) of 4n(*G. arboreum* × *herbaceum*).

Individual multivalents were classified according to their shapes in the hexaploids *G. hirsutum* (S4025 and S5082) × *raimondii* and (*G. hirsutum* × *thurberi*) (Table 3). No differences were observed between 6n(*G. hirsutum* S4025 × *thurberi*) and 6n(*G. hirsutum* S4025 × *raimondii*), but 6n(*G. hirsutum* S5082 × *raimondii*) differed from 6n(*G. hirsutum* S4025 × *raimondii*) (P ca. 0.02). In 6n(*G. hirsutum* S5082 × *raimondii*) the largest difference from the other hexaploids was a decrease in the proportion of zigzag quadrivalents which require only three chiasmata and an increase of the types which require four. Unfortunately, chiasma counts were not made to see if this hexaploid had an increased number of chiasmata.

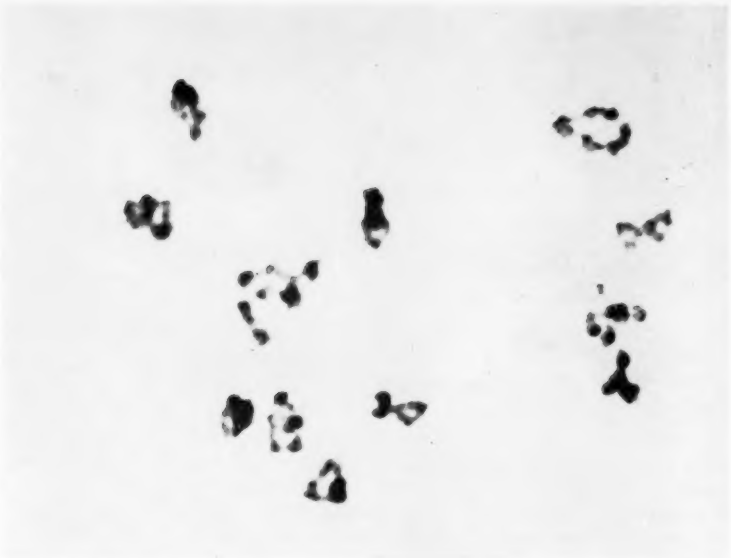


FIGURE 5.—Photograph of a PMC of 4n(*G. arboreum* × *herbaceum*) with complete conjugation. (11 IV + 1 VIII). ca. 2000 ×.

TABLE 3

Shapes of multivalents in hexaploids and their frequencies

No. of PMC's	Multivalent shapes													Total no. of multivalents
	A*	B	C	D	E	F	G	H	I	J	K	V	VI	
6n(*G. hirsutum* S4025 × *thurberi*)														
31	5	0	2	12	31	25	7	18	0	3	4	0	3	110
6n(*G. hirsutum* S4025 × *raimondii*)														
39	9	1	6	41	43	46	13	29	2	6	0	0	8	204
6n(*G. hirsutum* S5082 × *raimondii*)														
43	9	0	6	46	71	77	11	16	1	5	0	2	5	249

* For line drawings of these types see Figure 7.

Chiasma counts: Under the two methods of classification (see explanation above), the bivalent in Figure 3A would be counted in *method a* as having two chiasmata and in *method b* as having four. Ranges and mean numbers of chiasmata of both methods are given in Table 4. The two methods of counting chiasmata were tested for homogeneity for each amphiploid. In 6n(*G. hirsutum* S4025 × *thurberi*) and 6n(*G. hirsutum* S4025 × *raimondii*), both methods gave similar results, and the two hexaploids did not differ from each other. Also, no differences were obtained between the two methods for 6n(*G. hirsutum* SM4 × *raimondii*)

TABLE 4

Chiasma frequencies in Gossypium amphiploids

Amphiploids	No. of PMC's	Range of chiasmata per PMC		Mean chiasmata per PMC	
		a*	b†	a*	b†
6n(*G. hirsutum* S4025 × *thurberi*)	12	58–71	60–73	67.5	68.8
6n(*G. hirsutum* S4025 × *raimondii*)	16	63–73	63–78	67.9	70.2
6n(*G. hirsutum* SM4 × *raimondii*)					
Plants F and J	7	66–72	66–74	68.7	70.1
Plant D	9	66–74	71–82	70.6	76.0
6n(*G. barbadense* × *raimondii*)					
4/7/55	11	66–73	67–74	69.7	70.7
4/22/55	6	61–71	63–73	64.8	67.2
4n(*G. arboreum* "Sanguineum" × *anomalum*)	19	41–50	41–52	45.6	47.6
cold treated 29 days	11	44–50	45–53	47.0	48.5
4n(*G. herbaceum* × *anomalum*)	26	42–50	42–54	46.0	47.2
4n(*G. thurberi* × *raimondii*)	7	41–43	41–46	42.6	44.4

* Counts based on the assumption of a maximum of one half chiasma per chromosome arm.
† Counts based on apparent types of chiasmata as described in text.

plants F and J, but a highly significant difference was observed for plant D. When plants F and J were compared with D, the number of arms held together by chiasmata was the same, but the number of arms held together by more than one chiasma was much larger in the D plant. When the two dates of collection of 6n(*G. barbadense* × *raimondii*) were compared, a and b were significantly different.

Comparison of the a and b methods for each tetraploid showed significant differences only in (*G. arboreum* "Sanguineum" × *anomalum*) and (*G. thurberi* × *raimondii*). If the means from the two treatments of 4n(*G. arboreum* "Sanguineum" × *anomalum*) were compared separately with those from the other tetraploids, homogeneity was indicated except with 4n(*G. thurberi* × *raimondii*) which had fewer chiasmata per cell. The chiasma counts in both treatments of 4n(*G. thurberi* × *raimondii*) also differed from 4n(*G. herbaceum* × *anomalum*). Exposure of 4n(*G. arboreum* "Sanguineum" × *anomalum*) to cold for 29 days did not appear to affect the number of chiasmata formed.

Univalents: The mean numbers of univalents in 6n(*G. hirsutum* S4025 × *thurberi*) and 6n(*G. hirsutum* S4025 × *raimondii*) (Table 2) did not differ, but when both of these hybrids were compared with 6n(*G. hirsutum* S5082 × *raimondii*) highly significant differences were obtained. No differences were obtained when the two types of plants of 6n(*G. hirsutum* SM4 × *raimondii*) were compared with 6n(*G. hirsutum* S4025 × *raimondii*) but plants F and J of the former hybrids differed significantly from 6n(*G. hirsutum* S5082 × *raimondii*) while D did not. Comparisons of the univalent frequencies for the two collection dates from 6n(*G. barbadense* × *raimondii*) gave nonsignificant differences with each other and with 6n(*G. hirsutum* S4025 × *thurberi*) while with 6n(*G. hir-*

sutum S5082 × *raimondii*) the difference in the number of univalents was significant for April 7 and highly significant for April 22.

Microcytes: The average microcyte frequency per 100 tetrads was determined monthly for three different anther positions (Table 5). When these averages were

TABLE 5

*Average number of microcytes per 100 tetrads in hexaploids as influenced by anther position**

	Anther position			No. of tetrads counted
	Top	Middle	Bottom	
6n(*G. hirsutum* S4025 × *thurberi*)	16	15	10	4265
6n(*G. hirsutum* S4025 × *raimondii*)	22	16	5	3006
6n(*G. hirsutum* S5082 × *raimondii*)	18	9	8	2317

* Abnormal tetrads were excluded from the totals.

combined, 6n(*G. hirsutum* S4025 × *thurberi*) had 14 microcytes per 100 tetrads, 6n(*G. hirsutum* S4025 × *raimondii*) had 17, and 6n(*G. hirsutum* S5082 × *raimondii*) had 11. The microcyte frequency in the three hybrids did not differ for the distal position of the anthers, but the middle and the bottom positions were significantly different. Monthly values were not compared because of the wide variations. Summing the values over all positions and dates, the three hexaploids were significantly different from each other with respect to this irregularity. In addition, abnormal tetrads were seen in most of the anthers: 2.97 percent in 6n(*G. hirsutum* S4025 × *thurberi*), 0.83 percent in 6n(*G. hirsutum* S4025 × *raimondii*), and 1.45 percent in 6n(*G. hirsutum* S5082 × *raimondii*). These abnormal tetrads were often irregular in shape, number of spores, or amount of cytoplasm contained in the spores.

Pollen counts: Determination of pollen fertility proved difficult because there were many different types of pollen grains and overlaps between these types. Before studying the variation between the hybrids, the range from anther to anther in an individual flower was examined. Ten anthers from 6n(*G. hirsutum* S4025 × *raimondii*) ranged from seven percent good pollen for an anther at the top of the staminal column to 82 percent at the bottom. Twenty anthers from two 6n(*G. hirsutum* × *thurberi*) flowers gave from zero percent to 36 percent good pollen. As there was such a large anther to anther variation in a flower, the anther positions on the buds were kept separate.

Comparisons of the three hexaploids, *G. hirsutum* (S4025 and S5082) × *raimondii* and *G. hirsutum* × *thurberi*, were made whenever possible at the three anther positions for a year. If the monthly values were combined, there appeared to be an increase in good pollen from the top to the bottom anthers (Table 6). When the three hybrids were compared for each of the anther positions, statistical tests showed highly significant differences. The amount of good pollen in 6n(*G. hirsutum* × *thurberi*) was almost always lower than in the *G. hirsutum* × *raimondii* hexaploids. Also, the *G. raimondii* hexaploid with the marker stock S4025 was generally not as fertile as the hexaploid with S5082.

TABLE 6

Variation in percent of good pollen in hexaploids: influence of anther position

	Percent good pollen Anther position			Average no. of grains per anther
	Top	Middle	Bottom	
6n(*G. hirsutum* S4025 × *thurberi*)	10.3	9.6	18.0	166.4
6n(*G. hirsutum* S4025 × *raimondii*)	17.5	26.0	47.4	234.5
6n(*G. hirsutum* S5082 × *raimondii*)	44.3	57.8	70.1	253.9

Seed development: Ovules in the hexaploids, *G. hirsutum* (S4025 and S5082) × *raimondii* and *G. hirsutum* × *thurberi*, were counted at anthesis in order to compare them with the number of good and aborted ("motes") seeds found in mature fruits. Fruits with three locules averaged slightly more ovules than those with four locules (Table 7). 6n(*G. hirsutum* × *thurberi*) had fewer ovules than either

TABLE 7

Number of ovules per locule in hexaploids at anthesis

	No. fruits with		Mean no. ovules per locules		
	3 locules	4 locules	3 locules	4 locules	Average
6n(*G. hirsutum* S4025 × *thurberi*)	15	5	6.8	6.4	6.7
6n(*G. hirsutum* S4025 × *raimondii*)	8	8	8.7	8.3	8.4
6n(*G. hirsutum* S5082 × *raimondii*)	2	5	8.8	7.6	7.9

6n(*G. hirsutum* S4025 × *raimondii*) or 6n(*G. hirsutum* S5082 × *raimondii*).

Mature fruits of the hexaploids, *G. hirsutum* S4025 × *thurberi* and *G. hirsutum* S5082 × *raimondii* were examined for differences in the numbers of mature seeds and motes (Table 8). Three locule fruits had more seeds and motes per locule

TABLE 8

Counts of seeds and motes in mature fruits (hand crosses of hexaploids with Upland)

No. of fruits with		Av. no. of seeds& motes per locule			Average no. seeds per fruit	Average no. motes per fruit	Percent filled seeds	Percent seeds germinating
3 locules	4 locules	3 locules	4 locules	Average				
6n(*G. hirsutum* S4025 × *thurberi*)								
13	1	6.6	6.0	6.5	8.1	12.0	40.2	96.5
6n(*G. hirsutum* S5082 × *raimondii*)								
10	33	7.5	6.9	7.0	5.5	20.8	20.9	91.2*

* 3 fruits omitted.

than four locule fruits in both hexaploids. 6n(*G. hirsutum* S4025 × *thurberi*) had fewer seeds and motes per locule than 6n(*G. hirsutum* S5082 × *raimondii*), but was twice as fertile. Germination of seeds was high in both hexaploids, although 6n(*G. hirsutum* × *thurberi*) was slightly better.

DISCUSSION

Many conditions may modify the relationship between multivalent formation and segregation in polyploids. LITTLE (1945) lists five factors affecting segregation: mode of pairing, multivalent formation, nondisjunction, chiasmata, and position of the gene on the chromosome in relation to the centromere. Of these, the mode of pairing in an amphiploid is related to the amount of differential affinity between the chromosomes or the amount of heterogenetic or homogenetic pairing.

An amphiploid will give different segregation ratios on backcrossing to a parent species depending on the homologies between the chromosomes of the two species. If pairing is only homogenetic, then no segregation will result; and with random chromosome segregation 5:1 gametic ratios will be obtained. Between these two extremes other segregation frequencies will occur depending on the degree of preferential pairing. If it is assumed that all nonpreferential pairing will result in multivalent formation, then it is possible to calculate the amount of segregation based on observed multivalent frequencies.

In the hexaploid and tetraploid cottons studied there are 13 groups of four partially homologous chromosomes. Thus, with 13 multivalents, 5:1 ratios or 1/6 recessive segregants are expected. If an amphiploid averages only five multivalents, then for any specific group of chromosomes containing a factor under study there is a 5/13 chance of obtaining a quadrivalent and $5/13 \times 1/6$ chance of obtaining a recessive segregant for a factor located in the chromosomes involved. These calculations were repeated for 0, 1 . . . 13 multivalents expected for these cotton amphiploids, and the calculated values were plotted in Figure 6 which resulted in the straight line OG. Several assumptions had to be made in these calculations: a. Quadrivalents are distributed at random among the thirteen potential quadrivalents; b. Quadrivalents disjoin at random; c. Bivalents result from pref-

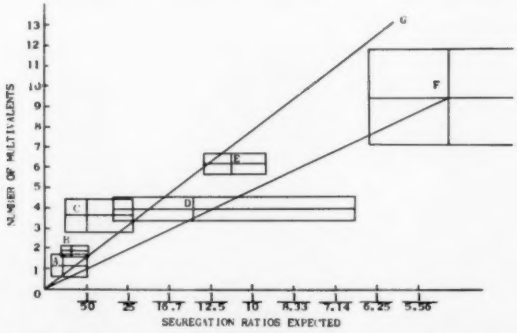


FIGURE 6.—Relationship between multivalent formation and segregation ratios. A. 6n(*G. hirsutum* × *anomalum*). B. 4n(Old World × *anomalum*). C. 6n(*G. hirsutum* × *thurberi*). D. 4n(*G. thurberi* × *raimondii*). E. 6n(*G. hirsutum* × *raimondii*). F. 4n(*G. arboreum* × *herbaceum*). Cytological data by the author with the exception of A, taken and corrected from IYENGAR (1944b). Genetical data from GERSTEL (1956) and GERSTEL and PHILLIPS (1957).

erential pairing; d. Univalents do not affect segregation; and e. There is no gametic elimination. Additional assumptions had to be made for the (2ADD) hexaploids: f. Quadrivalents are formed only from D chromosomes and are not A-D quadrivalents; and g. There are no A quadrivalents.

The mean numbers of observed multivalents and gametic segregation ratios for the various amphiploids are given in Table 9. The segregation ratios are averages obtained by GERSTEL (1956) and GERSTEL and PHILLIPS (1957) in testcrosses with recessives for one to several loci from each of the listed allopolyploids. Segregation and cytological studies were undertaken in the same plants except for the cytological data on 6n(*G. hirsutum* × *anomalum*) taken and corrected from

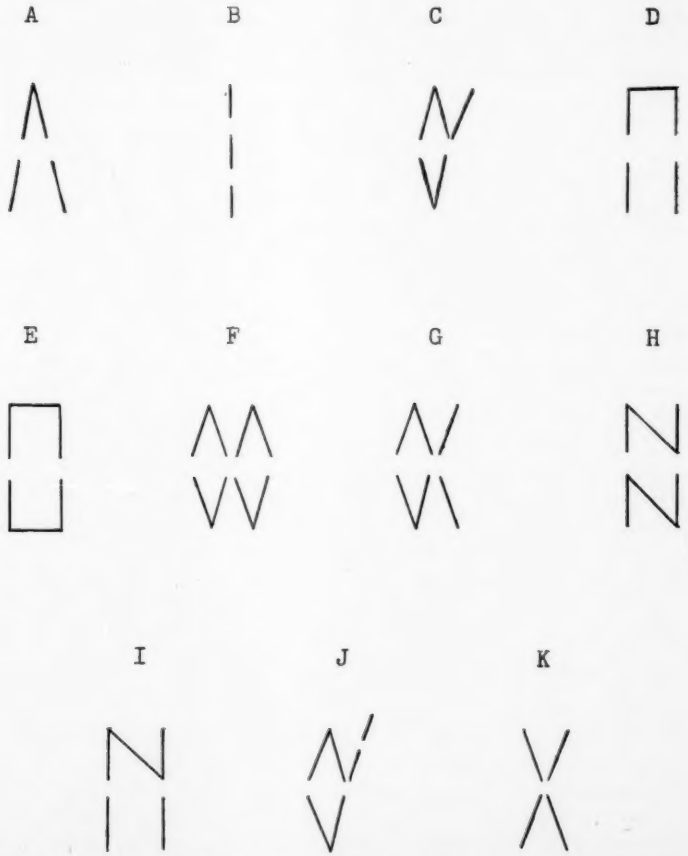


FIGURE 7.—Line drawings of the various shapes of multivalents found in the hexaploids.

TABLE 9

Average number of multivalents per pollen mother cell and segregation ratios in Gossypium allopolyploids

	Multivalents*			Segregation*	
	Number of PMC's	$\bar{x}$	$s_{\bar{x}}$	Number of plants	Gametic ratios
1. 6n(*G. hirsutum* × *anomalum*)	30	1.17±0.18		1,149	113.9:1
2. 4n(Old World × *anomalum*)	154	1.90±0.10		2,558	76.5:1
3. 6n(*G. hirsutum* × *thurberi*)	44	3.61±0.28		676	47.2:1
4. 4n(*G. thurberi* × *raimondii*)	16	3.94±0.19		167	12.9:1
5. 6n(*G. hirsutum* × *raimondii*)	108	6.16±0.19		2,744	10.1:1
6. 4n(*G. arboreum* × *herbaceum*)	12	9.42±0.69		702	4.1:1

* Cytological data were obtained from pollen mother cells by the author except for 1, taken and corrected from IYENGAR 1944b. Segregation data for amphiploids 1, 3, 5 were from the female; number 4 from the male; and 2 and 6 from both the male and female (GERSTEL 1956 and GERSTEL and PHILLIPS 1957). These segregation data were average gametic ratios obtained for one to several loci from each amphiploid.

IYENGAR (1944b). A point was plotted (Figure 6) for each amphiploid to represent both the genetical and cytological observations. Confidence limits were shown as a cross through each point and a rectangle was drawn to represent the 99 percent confidence limits. With perfect correlation a point should lie on line OG. If chance fluctuations were present, then the line should intersect the confidence limits. Line OG crossed the confidence limits of three of the six amphiploids while the other three were either off the line or the line crossed only a small portion of the rectangle. It was therefore necessary to attempt an explanation of these deviations and to ascertain how they might affect the prediction of segregation frequencies from observed numbers of multivalents.

Segregation of 4n(*G. arboreum* × *herbaceum*) was like that of an autotetraploid but there were fewer than the expected 13 multivalents. Although cytological analysis of this amphiploid was difficult because of the high number of multivalents, the most likely source of deviation arose from nonpreferential bivalents. Since this type of pairing seemed important here, it was probably also occurring in the remaining amphiploids and would invalidate assumption "c." used in calculating line OG. Therefore, a new line OF was drawn through the origin at one end and point F from 4n(*G. arboreum* × *herbaceum*) at the other (Figure 6) assuming that with the lower number of multivalents in the other amphiploids a proportionately lower number of nonpreferential bivalents would occur.

The divergence of point C, representing the *G. hirsutum* × *thurberi* hexaploid, must have had a different cause since in this case the number of multivalents was proportionately too high. Since 6n(*G. hirsutum* × *raimondii*) conformed better with expectation, a discussion of the assumptions from the viewpoint of the differences between the two hybrids may throw some light on the cause for the unexpected behavior of 6n(*G. hirsutum* × *thurberi*). As noted above, BROWN and MENZEL (1952) did not observe a significant difference in multivalent frequencies between these two hexaploid combinations.

It was assumed (a, above), that the quadrivalents were distributed at random among the thirteen potential quadrivalents in both hexaploids. The quadrivalents in 6n(*G. hirsutum* × *raimondii*) when tested by means of the binomial formula appeared randomly distributed and the probability of a quadrivalent being formed from any of the 13 groups of four chromosomes was 0.50. Similarly, quadrivalent formation in 6n (*G. hirsutum* × *thurberi*) also seemed to be at random since it also could be fitted to a binomial, but with a lower probability for any one quadrivalent to be formed, namely 0.31. Thus, the two hexaploid combinations do not seem to differ with respect to assumption a.

The triploids of these two hexaploids should provide a certain amount of information on the possibility of A-D pairing. A large amount of A-D pairing could be reflected by the number of trivalents and quadrivalents in the triploids and could result in a higher number of multivalents in the hexaploids. But multivalents in the triploids were infrequent (Table 1); thus, A-D pairing might be assumed to be negligible and would not increase the number of observed multivalents. However, in a three-species hybrid, (AD-A_2D_1), MENZEL and BROWN (1954) found that there was a considerable amount of A-D pairing.

Nondisjunction of quadrivalents which might affect segregation could occur occasionally as was shown by the presence of exceptional plants in the offspring of *G. hirsutum* × *raimondii* hexaploids (GERSTEL 1956). The rarity of these plants, however, indicated that either nondisjunction was low or else the gametes containing the products of nondisjunction did not survive. WEBBER (1934) reported that the quadrivalents in the cultivated American cotton disjoined regularly two by two, while according to BEASLEY (1942) quadrivalents separated in a 3:1 manner more than 13 percent and less than 50 percent of the time. Thus, nondisjunction may have occurred in the hexaploids to a limited extent but probably did not affect the ratios appreciably.

Zygotic or gametic elimination might also affect segregation. Gametes with an unbalanced chromosome set resulting from lagging chromosomes and subsequent microcyte formation could be lost. Univalents were rare in the amphiploids and comparison between 6n(*G. hirsutum* S4025 × *thurberi*) and 6n(*G. hirsutum* S4025 × *raimondii*) showed no difference in this respect. The higher number of univalents in 6n(*G. hirsutum* S4025 × *raimondii*) than in 6n(*G. hirsutum* S5082 × *raimondii*) could be attributed to more trivalents and pentavalents. The two hexaploids, *G. hirsutum* S4025 × *thurberi* and *G. hirsutum* S4025 × *raimondii*, which had the highest number of univalents also had the highest number of microcytes. It did not appear that these variations affected the segregation of one hexaploid more than another.

Elimination of gametes through pollen and ovule sterility might also affect the segregation ratios. The *G. hirsutum* × *thurberi* hexaploid usually had poor pollen compared with 6n(*G. hirsutum* × *raimondii*). In contrast to the pollen studies, ovule investigations indicated that 6n(*G. hirsutum* × *thurberi*) was twice as fertile as 6n(*G. hirsutum* × *raimondii*). Since most of the segregation studies had used the hexaploids as females and the cytological work was done with PMC's

(Table 9), there was a possibility of differences between megasporogenesis and microsporogenesis. To investigate this point, GERSTEL (1956) and GERSTEL and PHILLIPS (1957) compared the output of recessive (*aa*) and doubly dominant (*AA*) gametes by certain hexaploids (*AAaa*). Deviation from a 1*aa*: 1*AA* ratio would have indicated selective elimination. For the anthocyanin character in 6n(*G. hirsutum* × *thurberi*) the two classes were almost equal, but there were some discrepancies with respect to the leaf shape character. Thus, gamete elimination may have contributed to the aberrant behavior of 6n(*G. hirsutum* × *thurberi*). It should also be considered that the gametes containing one dose of the dominant and one dose of the recessive markers might have a selective advantage.

If the differences found in the *G. hirsutum* × *thurberi* and the *G. hirsutum* × *raimondii* hexaploids reflected differentiation between the chromosomes of *G. thurberi* and *G. raimondii*, then 4n(*G. thurberi* × *raimondii*) should also show preferential pairing and consequently have a lower number of multivalents than 4n(*G. arboreum* × *herbaceum*). The number of multivalents of the former was lower but could reflect a difference in the number of chiasmata which in turn could be influenced by the chromosome size since the smaller D chromosomes might form fewer multivalents than the A's. DARLINGTON (1929) found that quadrivalent formation in tetraploid hyacinths was highest in the long chromosomes and lower in shorter chromosomes. Unfortunately, only three fully analyzable PMC's were obtained from a 4n *G. thurberi* plant which could serve as a comparison between the number of multivalents possible in an autotetraploid D species and the essentially autotetraploid 4n(*G. arboreum* × *herbaceum*). These three PMC's averaged nine multivalents which is approximately the same as that of 4n(*G. arboreum* × *herbaceum*) (Table 9). Thus, it was assumed that chromosome size had little effect on multivalent formation in the A and D genomes. As with 4n(*G. arboreum* × *herbaceum*) the failure of point D (Figure 6) to lie on line OG probably was due to segregating bivalents. If line OF was used, point D was located near the line. From the segregation studies on the same hexaploids, GERSTEL (1956) concluded that *G. raimondii* is probably more closely related to *G. hirsutum* than is *G. thurberi*. The cytological evidence also supported this fact since the average number of multivalents was higher in 6n(*G. hirsutum* × *raimondii*) than in 6n(*G. hirsutum* × *thurberi*).

In amphiploids with *G. anomalum*, deviations from the theoretical lines were also observed. Confidence limits of the Old World × *anomalum* tetraploid (point B, Figure 6) were not intersected by line OF, and OG merely touched a corner of the confidence square. The lower amount of segregation in contrast to the other amphiploids might be expected from a tetraploid composed of two different genomes (A and B). Line OG crossed the confidence limits of 6n(*G. hirsutum* × *anomalum*) (point A, Figure 6) while line OF only crossed the corner of the confidence square.

Cold treatment of 4n(Old World × *anomalum*) plants showed little effect on the number of multivalents formed and on the chiasma frequency. The failure to observe differences might be the result of the small sample or else the plants

might have already recovered from the shock of transfer from normal to cold environment at the time the cytological samples were taken.

As all the other hexaploids were found to be homogeneous with respect to the number of multivalents, it was surprising to find that two collections in the *G. barbadense* × *raimondii* hexaploid differed, and the fixation with the lower number of multivalents had the higher number of chiasmata. Comparisons between multivalents and segregation, therefore, could not be made and would also indicate that wide variations might occur in an amphiploid and must be anticipated in selecting sample sizes to be used for predictions.

Since multivalents in the two different collections of 6n(*G. hirsutum* SM4 × *raimondii*) were similar to those of the other *G. hirsutum* × *raimondii* hexaploids, it was interesting to note in plant D that there was an increase of the number of bivalents having extra chiasmata (Table 4) which is very rare in cotton. Also in plant D unusual types of configurations were seen that were hard to interpret (Figure 3-B-H). Their shapes would suggest that there could have been a translocation close to the centromere which would account for the small bivalent and the unusually shaped bivalents and trivalents. Unfortunately, this plant set a low number of fruits and no segregation ratios were obtained for comparison.

In general, there was a relationship between the number of multivalents and the segregation ratios. Thus, hybrids composed of species that were more closely related tended to have more multivalents and, therefore, more segregation. It may be noted that line OF crossed the confidence limits of only two of the amphiploids while the other four deviated significantly. The four that deviated had in common lower amounts of segregation than would be predicted on the basis of the numbers of multivalents observed. Also, these four amphiploids were derived from species with two different genomes (intergenomic hybrids), while the two that fitted the line were hybrids between two species having the same genome (intragenomic hybrids). In conclusion, multivalent formation might be used to obtain an estimate of the amount of genetic segregation that could be expected from an amphiploid. Some of the deviations from expectation which occurred are discussed above.

SUMMARY

The behavior of the chromosomes of certain artificial amphiploids of the genus Gossypium was studied in order to establish the relationship between multivalent formation and genetic segregation. In hexaploid *G. hirsutum* × *raimondii* the mean number of multivalents was found to be 6.16 ± 0.19; nonsignificant differences occurred between different lines. Hexaploid *G. hirsutum* × *thurberi* had a lower multivalent frequency of 3.61 ± 0.28. The amphiploid *G. thurberi* × *raimondii* produced 3.94 ± 0.19 multivalents while 4n(*G. arboreum* × *herbaceum*) had the much higher average of 9.42 ± 0.69. In amphiploids of Old World cottons and *G. anomalum* there were only 1.90 ± 0.10 multivalents. The genetical data obtained previously from the same plants by Gerstel and Phillips were compared with these cytological results.

Theoretically expected segregation ratios were plotted in a graph against the frequencies of multivalents calculated under the assumption that all bivalents were the result of homogenetic pairing and that multivalents separated at random. This procedure gave a linear relationship. The observed mean values were then compared with the plotted line.

Certain deviations of observation from expectation were noted and discussed. A wide deviation of 4n(*G. arboreum* × *herbaceum*) from expectation could be explained as due to heterogenetic pairing of bivalents. In order to correct for this factor, a new straight line was drawn through the origin and the point representing 4n(*G. arboreum* × *herbaceum*).

Hexaploid *G. hirsutum* × *raimondii* gave a better fit to the first line than to the new line. 6n(*G. hirsutum* × *thurberi*) had too high a multivalent frequency to fit either line. This deviation was discussed in detail and the assumptions made when plotting the two lines as well as the data from chiasma frequencies, microcyte formation, fertility, and gametic and zygotic selection were examined. The last two factors might have influenced the observed segregation ratios.

The data from the remaining tetraploids did not closely fit either of the prediction lines, yet on the whole multivalent frequencies decreased and segregation ratios widened with increasing taxonomic distances between the species combined in allotetraploids.

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STUDIES OF LINKAGE IN POPULATIONS. II. RECOMBINATION BETWEEN LINKED INVERSIONS OF D. ROBUSTA

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LINKED gene arrangements of *Drosophila robusta* Sturtevant occur in non random combination in local populations of the species (LEVITAN 1954a, 1954b, 1955). Mathematical studies by WEINBERG (1909), ROBBINS (1918) and GEIRINGER (1944, 1948) predict that under panmixia linked loci will ultimately reach an equilibrium at frequencies representing populational independence between them. The speed at which the equilibrium is reached is directly proportional to the amount of recombination between the loci. If the effect of crossing over can be offset by selection, however, the population may not be able to reach this equilibrium, it may reach it at a slower rate, or, if the equilibrium has once been reached, the population may depart from it again. A first step in evaluating the factors responsible for the observed departures from randomness demands knowledge of the frequency of crossing over between the arrangements. Unfortunately, as CARSON (1953) and the writer have noted, independently, the extent of recombination between these inversions cannot be predicted from the chromosome distance between them. Instead, it must be determined empirically in each instance under a variety of background karyotypic conditions. This paper presents the available data concerning the arrangement combinations found locally. Brief preliminary reports of the data have appeared elsewhere (LEVITAN, 1953a, b; MASSIE and LEVITAN, 1956).

The data also bear on the problem of interchromosomal influences on crossing over. Despite recent renewed interest in this phenomenon (SCHULTZ and REDFIELD 1951; CARSON 1953; LEVINE and LEVINE 1954; REDFIELD 1955) its mechanism remains puzzling. In addition to supporting the structural and genotypic factors postulated by these authors, some of the data obtained here suggest a role of position effects as well (after STEINBERG and FRASER 1944).

MATERIALS AND METHODS

The data presented here were accumulated while determining the frequencies of the gene arrangements of *D. robusta* in two local woods. The collection and analysis methods used have been described elsewhere (LEVITAN 1951a, b, and 1955). While analyzing diapausing females in the fall of 1950, it was noted that the salivary glands which demonstrate that a collected female is heterozygous for inversions in both arms of a chromosome also provide evidence concerning the

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frequency of crossing over between the inversions. This happens because the method of analysis is essentially identical to a linkage testcross (see CARSON 1953 for a detailed description of the crossover study method). An attempt was made, therefore, to study the salivary glands of as many larvae from these diapausing females and their daughters as time would permit. The data fell into fairly clear patterns. The data suggested that concentration on the second chromosome material would be the most efficient course. Hence, flies collected after 1950 contributed to the X chromosome data only to the extent that each was smeared for the frequency analysis (usually eight to 12 larvae). For the second chromosome study, however, additional smears also were made. Whether time or the analysis slide was the limiting factor, the procedure was essentially random; that is to say, the amount of data obtained from individual females was generally not determined by its relationship to previous results. A check on this assumption is the following fact: the amount of data accumulated for various karyotype combinations turned out to be roughly proportional to their frequency in the natural population. Occasional bias appeared, however, as will be pointed out in the text.

Obviously the age of the collected females or the temperatures they had experienced in nature could not be controlled. This may detract somewhat from the validity of comparisons of these data to results obtained under more controlled conditions. In several instances however, data were obtained over the entire life span of (a) females which had obviously hatched in nature only a few hours prior to collection, and of (b) their daughters which hatched in the laboratory. The similarity of the results obtained from larvae produced by a female when she was young and when she was old, and the similarity of these results to data from females of unknown age, indicate that age is probably a negligible factor here. Also, the data resemble the results obtained by CARSON (1953) who had good enviromental controls.

Nomenclature

The gene arrangements of *Drosophila robusta* were first described by CARSON and STALKER (1947). They designated one arrangement of each chromosome arm as the "Standard" arrangement and named it for the arm: XL, XR, 2L and so forth. Other arrangements, which usually differed from the Standard by one-step inversions, were named by adding a number to the arm name; thus, for example, the four arrangements they found in the right arm of the X chromosome were named XR (the "Standard" arrangement), XR-1, XR-2 and XR-3. CARSON (1953) simplified these terms to identify whole structural karyotypes. When it was clear which chromosomes were being discussed, he substituted the letter "S" for the Standard arrangement of each arm and designated the other arrangements by the Arabic numeral in its name. Thus the arrangements mentioned above would be S, 1, 2, and 3 of X-right. If the left arm of chromosome 3 be omitted (since it usually presents only the Standard arrangement), and the chromosomes are understood to be in the usual order (the X chromosome being considered the

first chromosome, etc.), the constitution of a female with XL/XL, XR/XR-2, 2L/2L-3, 2R-1/2R-1 and 3R/3R-1 can be stated as S/S, S/2; S/3, 1/1; S/1, or even more succinctly, in linkage form: SS/S2, S1/31, S/1.

LEVITAN (1955) found this shorthand terminology very convenient for showing the alternative linkage combinations of flies heterozygous for arrangement in both arms of a chromosome. Thus a fly with 2L/2L-3 and 2R/2R-1 could have 2L linked to 2R and 2L-3 to 2R-1 or it could have 2L linked to 2R-1 and 2L-3 to 2R. The shorthand notation shows the linkage situation at a glance; the fly with S/3 in 2L and S/1 in 2R is either SS/31 or S1/3S. SS/31 and S1/3S are called the alternative "linkage types" of chromosome 2 "karyotype" S/3, S/1.

The crossover regions

Table 1 is concerned with the relative lengths of the regions where crossing over between inversions may occur in the Blacksburg populations. As a further use of CARSON's shorthand notation the regions have been named (first column) to identify, first, the chromosome (Roman letter) and second, the left and right arm inversions of that chromosome which they bridge (Arabic numerals). Thus, X-1,2 refers to the space between arrangement XL-1 and XR-2; II-1,1 the space between 2L-1 and 2R-1; and so forth. The extent of these regions and the amount of chromosome covered by the inversions is indicated diagrammatically in Figure 2 of CARSON and STALKER (1947). Figure 1 of CARSON (1953) is more exact, but it does not show all the regions pertinent to this paper. CARSON (1955) has measured the relative lengths of the chromosome divisions in salivary gland smears. Columns two and three of Table 1 of the present paper are based on these measurements. They show the relative amounts of euchromatin occupied by the regions. Each of the regions includes part (or, in the cases of X-1,2, X-2,2, and II-1,1, *all*) of the pertinent heterochromatic centromere areas. These centromere areas are "collapsed" in the salivary chromosomes. Hence, the percentages in columns two and three are probably understatements of the extent of the regions in meiotic

TABLE 1

Comparison of the amounts of salivary gland chromosome euchromatin in the regions where crossing over may occur between inversions in Blacksburg, Virginia populations of Drosophila robusta*

Chromosome region†	Percent of the euchromatin in all chromosomes	Percent of the length of the chromosome
X-1,2	9.0	23.8
X-2,2	10.9	27.9
II-1,1	15.7	37.7
II-2,1	8.9‡	21.4‡
II-3,1		

* Based on CARSON 1955.
† Explained in text.
‡ II-21 differs from II-31 in containing more chromocentric heterochromatin

chromosomes. The error is particularly large for the X chromosome regions because the pericentromere heterochromatin constitutes a much greater portion of X than of II. This may be deduced from the fact that the X is smaller than II in the salivary glands but is larger than II in other cells (compare their relative sizes in Figures 1 and 2 of CARSON and STALKER 1947). Consideration of the heterochromatin also implies that, contrary to the figures shown, region II-2,1 is longer than II-3,1; *how much* longer cannot be determined.

RESULTS

Recombination in the X chromosome. Table 2 shows the amount of recombination observed between X chromosome arrangements under different karyotypic conditions in the autosomes. The scarcity of crossing over between the X chromosome arrangements under any conditions is very striking. With such a rarity of

TABLE 2

Crossing over between X chromosome inversions under various karyotypic conditions in the second and third chromosomes

Condition in X	Condition in 3-Right	Condition in Second Chromosome: Homozygous in both arms ♀♀*	n†	% recomb.	Heterozygous for inversions in one arm ♀♀*	n†	% recomb.	Heterozygous for inversions in both arms ♀♀*	n†	% recomb.	Total ♀♀*	n†	% recomb.
S S / 1 2	S/S	7	83	0.0	26	364	0.3	21	1044	0.1	54	1491	0.13
	1/1	8	89	0.0	11	113	0.0	1	71	0.0	20	273	0.00
		1‡	20	30.0							1‡	20	30.00
	S/1	11§	124	0.0	43	629	0.8	19	1157	0.8	73§	1910	0.73
		12	144	4.2							74	1930	1.04
S 2 / 1 S	S/S	3	27	0.0	13	414	0.0	1	100	0.0	17	541	0.00
	1/1	2	31	0.0	6	66	0.0	3	333	0.3	11	430	0.23
	S/1	12	121	0.0	23	304	0.3	3	123	0.0	38	548	0.18
S S / 2 2	S/S	2	18	0.0	15	175	0.0	8	458	0.7	25	651	0.46
	1/1	0			2	17	0.0	0			2	17	0.00
	S/1	4	47	0.0	14	145	0.0	13	449	0.7	31	641	0.47
1 S / 2 2	S/S	0			8	85	0.0	0			8	85	0.00
	1/1	1	8	0.0	4	126	0.0	0			5	134	0.00
	S/1	1	10	0.0	10	110	0.0	2	107	0.0	13	227	0.00
Summary:													
Region X-1,2	Homozygous	20	230	0.0	55	957	0.10	25	1548	0.13	102	2735	0.11
	S/1	24	265	2.26	66	933	0.64	22	1280	0.70	112	2478	0.85
Region X-2,2	Homozygous	3	26	0.0	29	403	0.0	8	458	0.66	40	887	0.41
	S/1	5	57	0.0	24	255	0.0	15	556	0.54	44	868	0.35

* Number of females tested.
† Number of larvae examined.
‡ Female #C775 described in text.
§ Other females of this constitution.

crossovers most of the apparent differences between the amount of recombination under different background conditions are of dubious significance. When the third chromosome is heterozygous for arrangement, however, recombination between XL-1 and XR-2 (region X-1, 2) is significantly greater than when the third chromosome is homozygous. One female (#C775) collected in 1950, of linkage type SS/12 in the X chromosome, with 2S/2S in chromosome 2 and S/1 in chromosome 3-right, showed an unusually large frequency of crossovers, six out of 20 larvae examined. Even were the data from this female removed as atypical, the frequency of recombination in this region when chromosome 3 is S/1 remains significantly greater than when chromosome 3 is homozygous ($P < .01$, using YATE's correction to minimize the chi-square). Superficially, this effect of the third chromosome seems confined to X chromosome linkage type SS/12. The data for linkage type S2/1S which are S/1 in chromosome 3 is not significantly different from the comparable SS/12 data, however, even if the "atypical" female *is* included in the SS/12 material. The apparent differences between SS/22 and 1S/22 (region X-2, 2, Table 2) may also be spurious, particularly since no recombinants were obtained from wild-caught females of either type. All of the crossovers between XL-2 and XR-2 were from SS/22 daughters of female number C1902, herself atypical in crossover performance (see below). 1S/22 females of this type were not available for study. The data are also inconclusive on possible differences between the effects of S/S and S/1 on chromosome 3 for this region (X-2,2).

Recombination in chromosome 2. Table 3 shows the amount of recombination observed between second chromosome inversions under various karyotypic conditions in the X and third chromosomes. Here very clear differences appear. Crossing over in chromosome 2 is generally more frequent when the X chromosome is simultaneously heterozygous for inversions in both arms than when the X chromosome is heterozygous in only one arm; it is least of all when the X chromosome is homozygous for arrangement in both arms. Under similar conditions in the X chromosome significantly more recombinants between the chromosome 2 inversions are recovered if chromosome 3-right is heterozygous than if it is homozygous. The results agree with the data CARSON (1953) presented for one of the karyotypes (S/1, S/1).

The data concerning recombination in 2 when the X is heterozygous for inversion in both arms are presented in detail (Table 4). Included also are pertinent data of CARSON (1953), MASSIE and LEVITAN (1956), and unpublished data of CARSON (quoted with his kind permission). Although there are many empty places in the table, it may be noted that in a number of instances the amount of second chromosome crossing over is the same for different double heterozygotes in the X chromosome. Of particular interest is the uniformity of recombination in the largest series available, that between 2L-1 and 2R-1 (females S/1, S/1 in 2) when 3-right is S/1 and X is SS/12, SS/22, 11/22, or 1S/32. It follows that combinations of inversions giving results which deviate markedly from the rest must have special interchromosomal effects. These effects are not attributable to

TABLE 3

Crossing over between second chromosome inversions under various karyotypic conditions in the X and third chromosomes (local data only)

Crossover region	Condition in 3-Right	X chromosome homozygous in both arms ♀♀*	n†	% recomb.	X chromosome heterozygous in one arm ♀♀*	n†	% recomb.	X chromosome heterozygous in both arms ♀♀*	n†	% recomb.
II-1,1	S/S	2	59	1.7	11	250	2.0	4	332	14.2
	1/1	3	29	0.0	1	8	0.0	0		
	S/1	1	60	3.3	8	236	6.4	12	704	34.4
II-2,1	S/S	1	8	0.0	6	119	0.8	5	247	14.2
	1/1	1	11	0.0	2	95	0.0	1	70	18.6
	S/1	2	235	1.7	5	151	6.0	6	207	38.2
II-3,1	S/S	9	291	0.0	9	346	0.3	23	1138	8.0
	1/1	4	75	0.0	8	385	0.0	3	335	10.1
					1‡	185	14.1			
	S/1	9	228	0.0	21§	826	2.2	22	961	29.3
					22	1011	4.4			
All	Homozygous	18	504	0.60	37	1203	0.58	36	2122	10.35
	Heterozygous	14	472	1.06	35	1398	4.94	40	1872	32.21

* Number of females tested.
† Number of larvae examined.
‡ Females ♀C1902 described in text.
§ Other females of this constitution.

degree of structural heterozygosity. Particularly noteworthy here are (a) the difference between the effect of SS/11, on the one hand, and the effect of all other X chromosome karyotypes on recombination between 2L-1 and 2R-1, and (b) the different effects of X chromosome karyotypes S2/1S and SS/12 on recombination between 2L-3 and 2R-1. The odd result from SS/11 ("a" above) is susceptible to a genotypic explanation of the sort apparently envisaged by LEVINE and LEVINE (1954, 1955), namely, that a particular combination of genes trapped in the heterologous arrangements is responsible. The combination of alleles in SS/11 may not be completely repeated in any other X chromosome karyotype studied. S2/1S and SS/12 should contain the very same genes, however, because the very same inversions are involved. Different effects of S2/1S and SS/12 suggest, therefore, that the relative *position* of the genes, as well as their nature and zygotic condition, is involved in the interchromosomal effect. This point seems worthy of further study, especially since similar "positional effects" seem to have adaptive value (LEVITAN 1954a, 1955, 1957). (To differentiate these from the usual position effect DOBZHANSKY (1955) would dub them "organization effects").

Detracting from some of the above arguments is the fact that they are based on data from a small number of females. It was observed from time to time that certain females gave results differing widely from others with the same karyotypic conditions. One such "atypical" female, No. C775, was described with the X

TABLE 4

Crossing over between left and right arms second chromosome inversions under differing forms of double heterozygosity for inversion in the X chromosome

Crossover region	Karyotype in chromosome 2	Background karyotype in 3-Right	Background condition in the X chromosome: S S / 1 2			S 2 / 1 S			S S / 2 2			1 S / 2 2			1 1 / 2 2			S S / 1 1			1 S / 3 2		
			♀♀	n	%R	♀♀	n	%R	♀♀	n	%R	♀♀	n	%R	♀♀	n	%R	♀♀	n	%R	♀♀	n	%R
		S/S	4	332	14.2	0			3	300	12.0[f]	0			4	332	19.3[f]	8	700	4.4[f]	0		
II-1,1	S/1, S/1	1/1	0			0			4	400	7.5[f]	0			2	200	6.5[f]	0			0		
		S/1	11	691	34.2	0			6	486	34.2[h]	0			12	1150	34.3[f]	3	300	9.0[f]	3	150	29.3[g]
			1[e]	10	10.0[a]				2[e]	187	13.9[a]												
		S/S	1[d]	39	17.9[b]	0			1[d]	11	9.9[a]	0			0								
	S/2, S/1 (A)		2	49	16.7				3	198	13.6[a]												
II-2,1	or	1/1	1	70	18.6[a]	0			0			0			0								
	1/2, S/1 (B)								2[e]	34	29.4[a]												
		S/1	0			2	18	27.8[a]	1[d]	40	30.0[b]	1	115	45.2[b]	0								
									3	74	29.7												
		S/S	12[e]	510	6.3[a]																		
	S/3, S/1 (A)		3[d]	151	0.7[a]	1	100	2.0[a]	7[e]	377	14.9[a]	0											
II-3,1	or		15	661	5.0[a]																		
	1/3, S/1 (B)	1/1	0			2	322	9.9[a]	0			0											
		S/1	5[e]	282	26.5[a]																		
			21[d]	1233	17.4[a,j]	2	188	46.8[a,k]	10[e]	244	31.6[a]	1	9	0.0[a]									
			11[e]	894	18.0[b,j]	1	41	48.8[b,m]															
			37	2409	18.6	3	229	47.1															

Explanation of symbols in the heading: ♀♀ refers to number of females tested; n, to number of larvae examined; R, percent recombinations observed.

[a] Data from females with alternative karyotype "A" in second column.

[b] Data from females with alternative karyotype "B" in second column.

[c] Females tested were F_1 progeny of no. C1902.

[d] Females tested which were *not* progeny of no. C1902.

[e] Additional females tested which were not progeny of no. C1902; differ from the above (footnote "d") in alternative karyotype in second column.

[f] Data from CARSON (1953).

[g] Unpublished data of H. L. CARSON.

[h] Based on CARSON (1953) plus six recombinations out of 13 larvae from a female presumed to be S1/1S collected by the author; if this female was SS/11, the recombination frequency would be 34.4 percent.

[j] Based on MASSIE and LEVITAN (1956) plus prior data of the author.

[k] Based on 16 recombinants in 43 larvae from one SS/31 female and 72 recombinants in 145 from another; if latter female were S1/3S, this figure would be 47.3 percent.

[m] This result assumes linkage type of the female is 11/3S; were she 1S/31 the result would be 51.2 percent.

chromosome crossovers. Another example, No. C1902 was 12/22 in X, 2S/31 in 2, and S/1 in 3; the homozygosity in X-right was checked by over 20 larvae. She produced 26 recombinants out of 185 offspring (14.1 percent); 12 other females heterozygous in X-left, homozygous in X-right, and S/1 in 3 produced only four recombinants between 2L-3 and 2R-1 in 352 offspring (1.1 percent). Such heterogeneity is expected by chance less than once in 10,000 trials. Obviously, "atypicality" can be diagnosed only when a large enough series of females is available. To put it another way, the number of females tested may be as important in this type of study as the total number of offspring counted. Although the point could not be checked adequately in this study, there was some evidence that such variability between individuals is sometimes greater than variability between strains. Nine daughters of three wild females were all SS/12 in the X chromosome, SS/11 in chromosome 2, and S/1 in chromosome 3. The two daughters of one female produced 18 second chromosome recombinants in 64 larvae and 12 in 421, respectively; the corresponding recombination frequencies from three daughters of the second wild female were 45/104, 14/64, and 14/39, respectively; and those from four daughters of the third strain were 24/60, 23/90, 22/51, and 14/44. Analyzing by individuals gives $X^2 = 16.84$ for 8 degrees of freedom, P between .01 and .02, whereas analysis by strains gives $X^2 = 1.32$ for 2 degrees of freedom, P between .50 and .70. In a study by MASSIE (MASSIE 1955; MASSIE and LEVITAN 1956) both strain and individual heterogeneities occur. It is regrettable that these factors cannot be weighed properly in many publications.

The atypical females caused another bias factor. The oddity of their early results induced a special effort to examine as many of their offspring as possible, a deviation from the usual randomness of procedure. Selection of bottles from which to collect larvae for smear preparations also occurred when crossovers were so plentiful that the linkage type of a collected female was in doubt (e.g., some of the females mentioned in the footnotes to Table 4).

The karyotype of female No. C1902 suggested an experiment on interchromosomal effect. Since she had been mated to Standard males, some of her daughters were doubly heterozygous for inversion in chromosome 2, being SS/31 or, more rarely, SS/21. All were doubly heterozygous in the X chromosome, being either SS/12 or SS/22. Half would also be heterozygous in chromosome 3 (S/1), half homozygous (S/S). Crossover rates in the daughters would therefore give a comparison of the effects of these arrangements within a single strain. The SS/22 daughters (which contributed the bulk of the data for this type in Table 2) produced the only known laboratory crossovers between XL-2 and XR-2, none having been found by NELSON (1951), CARSON (1953), or by the author in other females. Five of the recombinant X chromosomes were S2; one, 2S. Otherwise, the X chromosome data followed the pattern in Table 2, the rarity of crossovers making detailed comparisons pointless. The second chromosome results of this experiment are denoted by footnote "C" in TABLE 4. Where comparisons are possible the daughters of C1902 tend to have higher recombination frequencies for region II-3,1 than do other females of the karyotype. Those with SS/22

in the X chromosome background produced more recombinants in this region than did those with SS/12, the difference being highly significant among those who are S/S in 3-right. The small amount of II-2,1 data is more ambiguous. Two tentative conclusions may be made: (1) a female can share with her daughters the capacity to boost recombination beyond the norm, this capacity thereby becoming a property of the "strain"; (2) differences between interchromosomal effects of karyotypes (X types SS/12 and SS/22 in this instance) apparently depend on factors inherent in the inversion combinations, since strain factors have been controlled in this experiment.

CARSON (1953) postulates an especially strong interchromosomal effect of the XL-2 XR-2 chromosome. The data from the 1902 F_1 experiment seem to fit this hypothesis. The rest of Table 4 discloses, however, that karoytypes lacking 22 in the X chromosome background often have as much or more second chromosome recombination than do karyotypes with 22.

DISCUSSION

Postulated influences on crossing over fall into two main categories: mechanical and physiological. Mechanical factors refer to gross structural relations of the chromosomes which promote or inhibit restitution *vs.* exchange, pairing, or other physical aspects of crossing over. Physiological factors on the other hand are conditions which presumably affect the cell metabolism and thereby the molecular structure, bondings, or fragility of the chromosomal strands. The physiological factors may be further subdivided into external, such as temperature, hormonal influences, etc., and internal, those intrinsically determined by the genotype of the cell.

Several items of data in this paper seem to favor the physiological thesis. On the positive side, genotypic factors could be inferred from (1) the data showing differences between X karyotypes SS/12 and S2/1S in influencing crossing over on chromosome 2, differences which seem to be attributable only to positional differences of the arrangements, and (2) the apparent transmission of superior crossing over ability from mother to daughters in strain #1902. On the negative side, several items argue against the primary action of structural factors. Particularly striking are the differences in crossover frequencies of the X and second chromosome crossovers as the number of heterologous chromosomes heterozygous for inversion is increased (Tables 2 and 3). In both chromosomes the euchromatin available for crossing over between right and left arm arrangements are nearly equal (Table 1). In both, also, few crossovers appear when the other chromosome are free of inversion heterozygotes. As inversion heterozygosity of similar magnitude increases on the heterologous chromosomes there is a strong increase in crossing over in chromosome 2 but a barely discernible one in the X chromosome; that is to say, the two chromosomes respond very differently to similar gross structural changes in the rest of the genome. Differences between chromosome regions in their response to interchromosomal effect have been reported by other workers (SCHULTZ and REDFIELD 1951; REDFIELD 1955). In their cases,

however, the regions showed differences in crossover frequency in the absence of heterologous inversions, which parallel the results under interchromosomal effect, thus indicating that the differences between regions were basic to the crossover process and not to the interchromosomal effect. No such parallel results occur in this instance. Another evidence against the structural factor hypothesis is shown by the data in Table 4 (and more clearly by the data of MASSIE and LEVITAN 1954), which are incorporated in this table). Note the "(A)" and "(B)" karyotype differentiations in column 2 and among the data in Table 4. These data show that chromosome 2 crossovers occur as frequently adjacent to a combination of overlapping (e.g., ⅓ of chromosome 2-left) or included (e.g., ½) inversions, as to simple ones (e.g., S/3 or S/2). Overlapping or included inversions might seem to cause greater difficulty in pairing than simple inversions.

These additions to the circumstantial evidence in support of the physiological factor hypothesis are not given with the intent to imply that structural factors can be ruled out, as an examination of the arguments for and against the hypotheses as related to interchromosomal effects will readily bear out (STEINBERG and FRASER 1944; SCHULTZ and REDFIELD 1951; REDFIELD 1955; CARSON 1953; LEVINE and LEVINE 1954). In essence, it can be seen that none of the arguments for one or another primary factor have been convincing, because the authors cannot agree whether supposed crucial implications of the various theories are indeed valid inferences (our knowledge of the basic processes of crossing over being so incomplete). In one of the cases cited above, for example, it might be asked whether overlapping inversions really do cause greater difficulty in pairing than do simple inversions. And even in a case where an implication may be acceptable and a postulated factor can be ruled out, who can guarantee that it is not an instance where one of several possible variables is silent?

It would seem more reasonable to recognize that there are elements of validity (evidence) in all of the studies, but that some of the factors are more predominant in some cases (or individuals) than in others. This could be formulated simply by considering crossing over another aspect of the phenotype and as such to be the product of genotype and environment. "Environment" would include mechanical factors as well as temperature, and "genotype" would include manifestations depending on position of the gene, special properties of heterochromatin and special regions of the chromosome, as well as the usually understood meaning of the term.

Such a formulation is particularly useful for the population geneticist; indeed it is implicit in work indicating that recombination is susceptible to natural selection and other evolutionary forces. In this regard there is a tempting analogy between the individuals found in this study with unusually high crossover ability and the identified carriers of mutator genes in populations of *Drosophila melanogaster* (IVES 1950).

Aside from such special cases, the population geneticist is usually less interested in the amount of crossing over that occurs in a given chromosomal region than he is in the frequency of crossover gametes in the population as a whole. This

latter condition is the factor which must be weighed against selection and other pressures in determining evolutionary changes in linkages. Table 5 contains a rough estimate of the rate at which recombination contributes to the supply of the various linkages in the Blacksburg area. The calculations are based on Tables 2 and 3 for crossover rates. For the frequencies of various karyotypes, the arrangement totals in an earlier paper (LEVITAN 1951b) are used, and, except for one special case noted below, the calculations assume validity of the Hardy-Weinberg equations and equality of alternative linkage types among females

TABLE 5

Estimated rate at which various inversion linkage chromosomes are produced by recombination in D. robusta *populations near Blacksburg, Va.*

Linkage*	Frequency of double heterozygotes producing it (in percent)	Estimated number per million female gametes
X chromosome:		
SS	7.0	166
S2	8.7	202
1S	7.0	166
12	9.0	206
2S	3.7	75
22	†	†
Second chromosome:		
SS or *S1*	3.2	943
1S or *11*	2.8	831
2S or *21*	2.4	711
3S or *31*	3.6	831

* For explanation of the notation see text.
† Very rare, possibly none in these populations; see text.

heterozygous for inversion in both arms of a chromosome. The writer has found that the Hardy-Weinberg law seems to be valid enough in these populations (LEVITAN 1955 and unpublished data), and that female second chromosome linkages are relatively equal (*op. cit.*). The X chromosome does present an excess of adult females SS/12 over those S2/1S (LEVITAN 1954b and unpublished data), but the difference between the linkage types is small, albeit significant, and it is ignored in the calculations. Differences between the possible linkage types involving XL-2 cannot be ignored, however, since two of these, S2/2S and 12/2S, are apparently completely absent among adult females (unpublished data), a consequence of the general rarity of linkage 2S (CARSON and STALKER 1949; unpublished data of the author). Accordingly the calculations assume that crossing over involving XL-2 occurs only in linkage types SS/22 and 1S/22.

Examination of Table 5 reveals that the rate at which recombination changes the supply of these linked gene arrangements varies from about seven to 95 per hundred thousand gametes. This figure is very similar to the frequency of changes typically produced in single loci by some of the higher rates of mutation and back mutation. It is also an order of magnitude that natural selection could

probably counteract without great difficulty, leaving little obstacle to selection's playing a dominant role in evolutionary changes involving these linkages. This is shown particularly well in the ability of 22 to survive, sometimes even increase in frequency (LEVITAN 1951b and unpublished data), even though some of the 22 must be depleted each generation by crossing over.

The implication of these points is that the population geneticist must deal with these linkages as integration systems, very nearly whole chromosome systems in some instances. Table 5 also shows that he must not be deflected from this viewpoint by the apparently high recombination frequencies in some individuals. This is seen in the relative similarity of the *populational* recombination rates in the X and second chromosomes even though in individuals the crossover rates in the second chromosome may be 100 or more times as great as in the X chromosome. The solution to the paradox lies in the relative frequency of double heterozygotes (column 2 of Table 5), which favors recombination in the X chromosome, and, on the other hand, the greater sensitivity of second chromosome recombination to degree of inversion heterozygosity in heterologous chromosomes which favors recombination in the second chromosome. The thought arises that this interplay of factors influencing recombination between the inversions is genotypically conditioned, the present situation resulting from evolutionary forces connected with the apparent adaptive character of the linkages (LEVITAN 1954a, b, 1955, 1957). Unfortunately, this attractive hypothesis seems very difficult to test, and probably must remain a speculation.

SUMMARY

The rate of crossing over between linked inversions of the X and of the second chromosomes of *Drosophila robusta* has been investigated by means of the salivary gland smear technique. Most of the data were obtained from individual females collected in two woods near Blacksburg, Virginia; a few data were also obtained from studies with the first or second generation progeny of these females.

The data show that the crossover rate between the X chromosome inversions is usually less than one percent. This rate is independent of the second chromosome karyotype present but is influenced by inversion heterozygosity on 3R.

The crossover rate between second chromosome inversions varies between less than one percent and almost 50 percent. It is influenced by the amount of inversion heterozygosity in both the X chromosome and the third chromosome. The rate also shows some proportionality to the length of second chromosome region involved, but it does not depend on whether the left arm is tied up by simple, included, or overlapping inversions.

Several instances of significant variation between individual females with similar karyotypic conditions were found. Some daughters of a female with unusually high crossing over also had a boosted crossover rate.

Crossing over between the proximal breakage points of 2L-3 and 2R-1 appears to be much greater when the X chromosome linkage type is S2/1S than when it is SS/12, suggesting a possible position effect.

From the populational viewpoint the rate at which the various linkage chromosomes are produced by recombination varies from about 75 per million female gametes for combination XL-2 XR to about 950 per million for 2L 2R (or 2L 2R-1). The discussion points out the similarity of these values to mutation rates and the ease with which natural selection could treat the linkages as integrated units at this level of populational recombination.

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GENETIC VARIATION IN AN ASEXUAL SPECIES, THE GARDEN STRAWBERRY[1]

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THE genetic variation, like other attributes, of a population is a product of evolutionary forces. These forces are shaped in part by the reproductive system of the organism. WRIGHT (1956) discussed selection in terms of the concept, outlined by him in various earlier papers, of multiple "adaptive peaks" in the potential array of genotypes of a species. In the process he drew a contrast between populations in which reproduction is exclusively biparental and ones in which it is ordinarily asexual but occasionally biparental. He indicates that with biparental reproduction and continuous selection of a constant sort in a relatively stable environment, the expectation in any single panmictic population is a set of coadapted genotypes. These genotypes would all be associated with a single adaptive peak (not necessarily the h'ghest) and coadapted in the sense that new genctypes derived by random intercrossing would have average value about equal to that of the parent genotypes. On the other hand, he pointed out that asexual reproduction allows strong selection among clones (individual genotypes) so that the emphasis is shifted from coadaptation to the excellence (fitness) of the single genotype in its own right. Thus surviving genotypes (clones) are more likely to be associated with different adaptive peaks and to exhibit less coadaptation.

The suggested contrast in coadaptation can be illustrated in terms of economic plants. Random intercrossing in an open-pollinated variety of corn yields a new generation about equal in value to the parental generation. However, random crosses among clones of strawberries, potatoes, sugar cane, etc. that have survived as commercial varieties yield progenies of markedly lower value than the parents.

The existence of multiple adaptive peaks is not absolutely dependent on presence of epistasis, but it is usually assumed that interactions of nonallelic genes will be a significant feature of any multiple peak system of genotypes. On this assumption WRIGHT's hypothesis suggests that a population obtained from random crossing among selected clones of an asexual species should exhibit (1) relatively large amounts of genetic variances, and (2) considerable epistatic variance. This manuscript reports and discusses evidence on the genetic variance

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in five quantative characters of such a population. Each of the five characters is related to productivity and hence may be assumed to have been selected for in the past.

A basis for characterization of genotypic variance was established by FISHER (1918) who, by definition, partitioned it into three components: (1) a portion associated with the average effects of genes (Later, 1930, he referred to this as the *genetic* variance. However, in conformity with general usage in this country we shall call it the additive genetic variance.), (2) a portion associated with dominance effects (allelic interactions) of genes, and (3) a portion associated with epistatic effects (nonallelic interactions) of genes. He provided a basis for inference about these components of variance by showing how the additive genetic and dominance variance contribute to correlations among relatives in random breeding populations. A logical scheme for subdivision of the epistatic variance has been provided by COCKERHAM (1954) and KEMPTHORNE (1954, 1955). At the same time they showed how the resulting partitions of the epistatic variance contribute to the correlations among relatives.

COMSTOCK and ROBINSON (1948, 1952) described procedures for estimating the additive genetic and dominance variances of plant populations. Two of these have been used effectively in studies of the genetic variation of grain yield and other traits in corn (ROBINSON *et al.*, 1949, 1955; Gardner *et al.*, 1953). Another, applicable with multiflowered plants, was used in this study. It had previously been used in work with *N. tabacum* (ROBINSON *et al.* 1954), and is closely analogous to the approach followed by HAZEL and LAMOREAUX (1947) in an investigation of genetic variation in age at sexual maturity in White Leghorn pullets.

Developments and inferences with respect to composition of the genotypic variance, in all the papers cited above, are premised on bivalent pairing at meiosis. Hence, it is important to note that, while the garden strawberry is believed to be an octoploid of probable interspecific origin (DARROW 1937), POWERS (1944) in studies of *F. ovalis* and *XF. ananassa* demonstrated essentially regular meiotic behavior of the diploid type.

The observation by MORROW and DARROW (1952) of strong inbreeding depressions when strawberries are self-fertilized indicates considerable nonadditivity in the action of genes affecting such characters as yield, vigor and berry size.

MATERIAL AND METHODS

Materials, field design and data. Forty clones from among those which might logically be employed as foundation stock in the North Carolina Agricultural Experiment Station breeding program were employed as parents. (Included were named varieties, unnamed selections and some clones obtained by one generation selfing of a variety or selection.) These were divided randomly into four sets of ten. Each set was then subdivided randomly into two groups of five and within each set every parent in one group was crossed with each in the other group. Hence had each cross been successful 25 progenies would have been obtained in each set. However, the crosses involving two parents were unsuccessful in the

sense that insufficient seed for the purposes of the experiment were obtained. The result was two sets of only 20 (instead of 25) progenies each. For convenience in discussion one group of parents in each set will be referred to as males, the other as females.

The progenies of each set were compared in a randomized block field design with four replications. Each progeny was represented in each replicate block by a plot made up of four "seedling squares", a seedling square being comprised of a seedling plant and runner plants derived from it. Each square was allowed to develop to an area two feet on a side, if the seedling had sufficient runnering potential, but was prevented by cultivation from becoming larger.

In addition to a plot of each of the 20 or 25 progenies of the set, several plots within which the four squares were initiated from single runner plants of the same clone were included in every replicate block of the experiment. Since genotype was constant within each of these plots, they provided data for estimation of intraplot environmental variance among squares. The clones used in these plots were, with one exception, a sample from the 38 parents of the experimental progenies. They included Albritton, Blakemore, Klonmore, Massey, Tennessee Shipper, Tennessee Beauty, and two unnamed selections.

All plants, both seedlings and runner plants from sample parents, were established in individual pots in the greenhouse. The seedlings were transplanted to the field on May 5, 1954 and the runner plants on June 5, 1954. Data were collected in the spring of 1955.

Data to be reported were taken on square size, number of berries and total weight (in grams) of berries. Ripe berries were harvested, counted and weighed at semiweekly intervals; weights and numbers being collected finally into seasonal totals for each square. A square grid subdivided by cross wires into nine equal parts was used in estimating square size. The grid was held just above the square so that the area of the square, in terms of subdivisions of the grid, could easily be estimated. The total area of the grid was smaller than four square feet (the maximum size of the plant square) by an amount equal to the area of each of the nine subdivisions. If the plant material covered an area larger than the grid, it was scored ten, otherwise less according to correspondence with the grid.

From the raw data, two more characters to be considered were computed. Average weight per berry was computed as the quotient of total weight divided by total number of berries per square. Weight of berries per unit area of plant material was computed for each square as the quotient of total weight of berries per square by square size. Weight per berry measures fruit size. Weight of fruit per unit square size provides a measure of productiveness that is somewhat different (and possibly more significant relative to horticultural utility) from weight of fruit per square. The latter is a partial function of the capacity of the genotype for vegetative proliferation, but in commercial use poor proliferation can be compensated by closer spacing when plants are set. Hence while proliferation ability is obviously of some value, high yields are possible without it; and a

genotype with high yield per unit area of plant material may be more valuable than one that develops more vegetative material but yields less per unit area.

Definitions of effects and symbols and analysis of data. A feature of the experiment that deserves consideration in interpretations is that all data were collected at one location in one year. Thus phenotypic measurements obtained pertain most directly to genetic effects defined with respect to the environments of the specific year-location of the experiments. Description of the analysis of the data and discussion of the results will be in that context.

Let the measure of any character for an individual square be represented as

$$P = x + m + f + mf + r + b + v$$

where x = the general mean of the character over the population of progenies of which those in the experiment were a sample and the population of environments associated with the year-location of the experiment,

m = the average effect of genotype for the offspring of a single male parent,

f = an effect defined similarly relative to a female parent,

mf = the difference between the average genotypic effect for members of a full-sib family and the sum of m and f for the parents of the same family,

w = the deviation of the effect of genotype of a single seedling from the average effect of genotype for the full-sib family,

r = average environmental effect for a replicate block,

b = the intrablock environmental effect of a plot, and

v = an intraplot environmental effect associated with a square plus the error of measurement.

The above assumes no nongenetic effects of parents, e.g. no cytoplasmic or other maternal effects.

The form of the analysis of variance for estimation of variance components was largely standard and will not be detailed. σ_v^2 was estimated with 192 degrees of freedom, as the intraplot variance among squares in the data from plots within which the separate squares were established asexually from a common clone. σ_w^2 was estimated as the difference between this estimate of σ_v^2 and the intraplot variance of squares established each from a different seedling.

RESULTS

Estimates. Variance component estimates are listed in Table 1. Estimates of σ_m^2 and σ_f^2 have been pooled into one estimate of genetic variance among half-sib family means denoted as σ_m^2. This appeared appropriate because (1) in the absence of maternal effects, the estimates of σ_m^2 and σ_f^2 have the same expectation and (2) the estimates themselves were compatible with the notion that σ_m^2 and σ_f^2 were equal in the material investigated.

As a preliminary basis for inference from the estimates of genetic variances

TABLE 1

Variance component estimates

Estimate	Square size	No. of berries	Total wt. of berries	Av. wt. of berries	Wt. of berries per unit square size
σ^2_m	.32	454	9,698	.16	155
σ^2_{mf}	.20	319	6,184	.02	69
σ^2_w	1.89	5,473	126,013	1.14	3340
σ^2_b	1.04	755	25,905	.06	575
σ^2_r	2.93	2,411	72,211	.53	4325

(σ^2_m, σ^2_{mf} and σ^2_w) consider the composition of these variances under the following assumptions.

(1) Regular diploid behavior at meiosis.

(2) No cytoplasmic or maternal effects.

(3) No correlation of genotypes at separate loci. This implies no linkage among genes affecting any single character or that, where linkage existed, the distribution of genotypes was as expected in the absence of linkage.

(4) That the distribution of genotypes in the parents was of the nature to be expected in a random sample from a random breeding population.

(5) No epistasis, i.e. that effect of variation in genotype at one locus is not a function of genotype at other loci.

When these assumptions are correct, there is no epistatic variance and the variances being considered are composed as follows:

$$\sigma^2_m = \frac{1}{4}\sigma^2_{10}$$

$$\sigma^2_{mf} = \frac{1}{4}\sigma^2_{01}$$

$$\sigma^2_w = \frac{1}{2}\sigma^2_{10} + \frac{3}{4}\sigma^2_{01}$$

where σ^2_{10} and σ^2_{01} are additive genetic and dominance variance, respectively. Now let

$$\sigma^2_i = \sigma^2_w - 2\sigma^2_m - 3\sigma^2_{mf}$$

Under assumptions listed above $\sigma^2_i = 0$. On the other hand, significant deviation of an estimate of σ^2_i from zero indicates nonvalidity of one or more of those assumptions.

Estimates of σ^2_{10}, σ^2_{01} and σ^2_i were computed as follows:[1]

$$\hat{\sigma}^2_{10} = 4\hat{\sigma}^2_m$$

$$\hat{\sigma}^2_{01} = 4\hat{\sigma}^2_{mf}$$

$$\hat{\sigma}^2_i = \hat{\sigma}^2_w - 2\hat{\sigma}^2_m - 3\hat{\sigma}^2_{mf}$$

They are listed, along with estimates of the total genotypic variance, in Table 2. The latter were obtained as

$$\hat{\sigma}^2_y = 2\hat{\sigma}^2_m + \hat{\sigma}^2_{mf} + \hat{\sigma}^2_w$$

TABLE 2

Estimates of genetic variances

Estimate	Square size	No. of berries	Total wt. of berries	Av. wt. per berry	Wt. of berries per unit square size
$\sigma^2_{10} = 4\sigma^2_m$	1.28	1,857	38,793	.64	622
$\sigma^2_{01} = 4\sigma^2_{mf}$	.80	1,276	24,738	.08	276
σ^2_i	.65	3,587	88,063	.76	2823
σ^2_y	2.73	6,720	151,594	1.48	3719

Accuracy of estimates. Errors of estimation are of two kinds: random and non random (the latter type of error is often called bias). Given appropriate data, the potential magnitude of random errors can be approximated by statistical methods. Non random errors, on the other hand, are functions of experimental procedure and usually cannot be detected by examination of the data. Conclusions concerning them hinge on judgment as to whether materials and procedures were such that the expectations of estimates were truly in accord with what is to be assumed for purposes of interpretation.

In this instance the nature of the findings raises the question of whether σ^2_r for the material from which it was estimated (the plots within which squares were genetically homogeneous) was equal, as assumed, to σ^2_r for the seedling squares. These two portions of the experimental material differed first in the way of origin of the plants from which squares were established and, second by one month in date of transplanting. On the basis of the experience of one of the authors (MORROW) and his observations of the experimental material during its development in the field, the authors consider the assumption of approximate equality of σ^2_r for all the material to be justified. (The issue had been discussed in detail prior to

[1] The symbol ^ indicates "estimate of".

PROFESSOR MORROW's untimely death.) This judgment receives some support from estimates of environmental variance due to plot differences which were available for both asexual and seedling portions of the material. These estimates were larger for the asexual material in the case of three characters and smaller in the case of two characters but did not differ significantly for any of the five characters.

Random errors were assessed in two ways. Four estimates of each genetic variance were obtained, one from each set of progenies grown. The variance among the four estimates provided an estimate of the standard error of the mean of the four estimates. The second method made use of the fact that where effects contributing to mean squares are normally distributed, the variance of a mean square is $2[EM]^2/d$ where (EM) is the expectation of the mean square and d is the number of degrees of freedom for its estimation. Since all estimates were obtained as linear functions of two or more mean squares, their standard errors could be estimated assuming normal distribution of all genetic and environmental effects and substituting observed mean squares for their expectations. Table 3 lists each variance estimate as a multiple of each of the two estimates of its standard error.

These figures indicate that so far as random errors were concerned, σ_y^2 was generally well estimated, estimates of σ_m^2 and σ_i^2 were moderately accurate in most instances, but estimates of σ_{mf}^2 were not large relative to their standard errors. Reference to Table 1 indicates that the latter fact is due in large part to comparatively low estimates in the case of σ_{mf}^2.

TABLE 3

*Estimates of genetic variances divided by estimates of their standard errors**

Character	Variance			
	σ_m^2	σ_{mf}^2	σ_i^2	σ_y^2
Square size	7.97	1.59	1.19	6.43
	2.79	1.09	1.11	5.90
No. of berries	2.38	1.80	6.22	10.27
	2.56	1.49	4.54	11.59
Total wt. of berries	5.37	1.77	6.26	16.55
	2.36	1.22	4.44	10.53
Av. wt. per berry	2.36	.50	3.89	5.78
	3.46	.58	4.62	11.02
Wt. of berries per unit square size	2.87	.53	1.62	3.31
	1.59	.91	3.64	6.21

* The first figure in each instance was computed using the standard error obtained from variation among the four separate estimates of the variance.

DISCUSSION

A two part hypothesis has been advanced (see introductory remarks) concerning the genetic variance to be anticipated in a population derived through random cross fertilizations in a species where reproduction is usually asexual so that strong selection among individual genotypes is a prime feature in the evolution of the species. The first part asserts that the total genetic variance will be large; the second, that there will be considerable epistatic variance. The hypothesis is predicated on assumption (as by WRIGHT, *op. cit.*) that interaction among nonallelic genes is fundamental and significant relative to the physiological dependence of phenotype on genotype. In this connection, it should be noted that nonallelic gene interaction does not by itself insure epistatic variance. Epistatic variance arises only when the population is genotypically variable at two or more loci involved in the same system of interacting genes.

The hypothesis restated above directs attention to the evidence concerning the magnitudes of total genetic variance and epistatic variance. In addition the estimates of dominance variance are pertinent in so far as they enable inferences concerning level of dominance in gene action.

The genetic coefficient of variation provides a reasonable measure of magnitude of the total genetic variation. It was computed as $100\sqrt{\hat{\sigma}_y^2}/\bar{x}$, where $\bar{x}$ is the observed mean for the character. The five coefficients are listed below.

Character	*Genetic C.V.*
Square size	34.4
No. of berries	48.1
Total wt. of berries	45.0
Av. wt. per berry	23.7
Wt. of berries per unit square size	31.8

A coefficient of size 20 indicates a range in genetic value from roughly 50 to 150 percent of the mean for the character, an amount of variation that cannot be viewed as small. By this criterion the estimate of total genetic variance was large for each of the five characters.

The genetic variance estimates presented are such that σ_y^2 is the sum of σ_{10}^2, σ_{01}^2 and σ_i^2. Consequently all information regarding their relative magnitudes is contained in the two ratios, $4\hat{\sigma}_m^2/\hat{\sigma}_y^2$ and $\hat{\sigma}_i^2/\hat{\sigma}_y^2$. These are as follows:

Character	$4\hat{\sigma}_m^2/\hat{\sigma}_y^2$	$\hat{\sigma}_i^2/\hat{\sigma}_y^2$
Square size	.47	.24
No. of berries	.28	.53
Total wt. of berries	.26	.58
Av. wt. per berry	.43	.51
Wt. of berries per unit square size	.17	.75

The most arresting aspect of these ratios is the uniformly large size of $\hat{\sigma}_i^2$ relative to $\hat{\sigma}_y^2$. Only in the case of square size is $\hat{\sigma}_i^2$ less than one half the estimate of total genetic variance.

If each of the assumptions listed in the preceding section were correct, σ_i^2 would be zero. Thus the evidence indicates that one or more of those assumptions is incorrect for the material investigated. It is easily demonstrated that the results are explainable in terms of the presence of epistatic variance. Retaining all assumptions except that of no epistasis and following COCKERHAM (1954) and KEMPTHORNE (1954) we obtain

$$\sigma_i^2 = \sum [1 - 4(\frac{1}{2})^{A+2D} + 4(\frac{1}{4})^{A} \quad 0^{D}] \, \sigma_{AD}^2$$

where summation is over all pairs of values of A and D such that $1 \leqslant (A + D) <$ (the number of loci affecting the character),

A is the number of times "additive" appears in the name of the variance component, and

D is the number of times "dominance" appears in the name of the variance component.

To exemplify,

when $A = 1$ and $D = 0$, $\sigma_{AD}^2 = \sigma_{10}^2$ = additive genetic variance

when $A = 0$ and $D = 1$, $\sigma_{AD}^2 = \sigma_{01}^2$ = dominance variance

when $A = 2$ and $D = 0$, $\sigma_{AD}^2 = \sigma_{20}^2$ = the "additive × additive" component of epistatic variance

when $A = 1$ and $D = 1$, $\sigma_{AD}^2 = \sigma_{11}^2$ = the "additive × dominance" component of the epistatic variance, etc.

Writing σ_i^2 term by term we have

$$\sigma_i^2 = \frac{1}{4}\sigma_{20}^2 + \frac{9}{16}\sigma_{30}^2 + \frac{49}{64}\sigma_{40}^2 + \frac{1}{2}\sigma_{11}^2 + \frac{3}{4}\sigma_{02}^2$$
$$+ (\frac{7}{8} \text{ or more}) \text{ (each of all other components of the epistatic variance).}$$

Remembering that the total genetic variance (σ_y^2) is the sum of the whole of all genetic variance components, it is apparent that the observed values of $\hat{\sigma}_i^2/\hat{\sigma}_y^2$ are consistent with the proposal that a major fraction of σ_y^2 is made up of epistatic variance components other than σ_{20}^2 (the "additive × additive" portion). It remains to note that the estimates of σ_{10}^2 and σ_{01}^2 considered relative to those of σ_i^2 and σ_y^2 are plausible under this explanation.

It is tempting to conclude without further ado that the large estimates of σ_i^2 are a reflection of considerable epistasis (in accord with the hypothesis outlined at the outset). However, discussion of other possibilities is in order.

Given allelic interactions (dominance) and quadrivalent associations at meiosis, σ_i^2 would take a positive value (KEMPTHORNE 1955). Intuitively, it appears the same would be true for other deviations from diploid meiosis. However, while we cannot be certain that his findings are representative of all cultivated strawberries, the assumption of diploid meiosis is supported by POWERS *(op. cit.)* and to our knowledge there is no evidence to the contrary.

Presence of cytoplasmic or other maternal effects would lead to underestimation of σ_i^2 and hence is not to be considered as a possible explanation of large estimates.

Assumption of no correlation of genotypes at different loci would be untenable if the parental stocks had all been taken from a population recently established by intercrossing a limited number of clones. Further it is easily demonstrated that such correlations may exert significant effects on genetic variances of the general sort being considered (MATHER 1949; COMSTOCK and ROBINSON 1952; HAYMAN 1954). However, in view of the sort of parental stocks employed the authors do not believe that correlations of genotypes as a consequence of linkage could have been sufficient to provide the explanation for results under discussion.

Finally, it is appropriate to inquire whether the large estimates of σ_i^2 may have been due to deviations of genotype frequencies in the parents from the expectation for a random sample from a random breeding population. The issue can be illustrated in terms of a simple situation where all genetic variance arises from one pair of alleles, the more favorable allele being completely dominant. Prior to any selection the expected genotype frequencies in a random breeding population are p^2, $2pq$ and q^2 where p and q are the frequencies of the two alleles. Whatever the actual genotype frequencies in a set of parents, they can be expressed as follows:

Genotype	*Frequency*
BB	$p^2 + fpq$
Bb	$2pq(1 - f)$
bb	$q^2 + fpq$

where f is the deviation, in fractional terms, of heterozygote frequency from $2pq$. Assuming these generalized genotype frequencies in the parents it can be shown that

$$\frac{\sigma_i^2}{\sigma_y^2} = \frac{-f(2+fp)}{2-p} \text{ and } \frac{4\sigma_m^2}{\sigma_y^2} = \frac{2(1+f)(1-p)}{2-p}$$

It is apparent that, if heterozygote frequency is in excess of $2pq$, then σ_i^2/σ_y^2 will be positive. An excess of heterozygotes would result from greater selective elimination of *bb* than of the other two genotypes. The next question is whether selection could disturb genotype frequencies enough to account for results observed.

Consider a population of zygotes from sexual reproduction in which the gene

frequencies are p_0 and q_0. Assuming random fertilizations in formation of these zygotes, the expected genotype frequencies are

BB	p_0^2
Bb	$2p_0q_0$
bb	q_0^2

In the extreme situation where all *bb* genotypes are eliminated by selection (and the other two genotypes remain in the ratio $p_0^2 : 2p_0q_0$ because with complete dominance their selective values are equal), the frequency of *B* goes to $1/(2-p_0)$ and f takes the value $-(1-p_0)$. The values of σ_i^2/σ_y^2 and $4\sigma_m^2/\sigma_y^2$ that would result if the remaining individuals were used as parents in the manner of the experiment reported, are easily computed. They are given below for various values of p_0.

p_0	$p=1/(2-p_0)$	$f=-(1-p_0)$	σ_i^2/σ_y^2	$4\sigma_m^2/\sigma_y^2$
.2	.56	—.8	.86	.12
.4	.63	—.6	.71	.21
.6	.71	—.4	.53	.27
.8	.83	—.2	.31	.23

Values very similar to those obtained from the data are seen in this table. In the real situation the genetic variance must have arisen from many loci rather than just one. However, in the absence of epistasis and with complete dominance and selective elimination of all recessive homozygotes at each locus, the result would be the same. The question that arises is whether all (or even very nearly all) recessive homozygotes would be eliminated by selection intensities that have prevailed if very many loci were involved. With no interlocus correlation of genotypes the proportion of individuals not homozygous for the recessive allele at any locus is $[1-(1-p_0)^2]^n$ where n is the number of loci. With no errors in selection the fraction selected would need to be $[1-(1-p_0)^2]^n$ if all recessive homozygotes were to be eliminated. This quantity becomes very small as n increases. For example, for $p_0 = .6$ and $n = 50$ it is less than .0002. Selection in strawberries is without doubt intense, but in view of the probable number of segregating loci and the errors in selection that must occur as a consequence of nongenetic variation, it seems inconceivable that locus by locus the relative frequency of homozygous recessives would be reduced by more than one half in the sort of clonal material represented by the parents used in this experiment. With complete dominance and no epistasis, the values of σ_i^2/σ_y^2 and $4\sigma_m^2/\sigma_y^2$ would then be as follows:

p_0	σ_i^2/σ_y^2	$4\sigma_m^2/\sigma_y^2$
.2	.15	.72
.4	.22	.56
.6	.21	.44
.8	.15	.27

Here σ_i^2/σ_y^2 is not as large as estimated and the largest values are associated with values of $4\sigma_m^2/\sigma_y^2$ larger than estimated. These values together with others (computed but not to be reported) obtained assuming both partial and over dominance have led the authors to conclude that selection as intense as reasonable to propose could hardly be the source of sufficient excess in heterozygotes to account completely for the observed ratios among the genetic variance component estimates.

Taken all together the considerations presented suggest that while the estimates of σ_i^2 may not have been due entirely to epistasis, they are probably indicative of considerable epistatic variance.

The ratios observed (Table 2) between estimates of the additive genetic and dominance variances will not be discussed in detail. In general the dominance variance appeared to be a little larger relative to additive genetic variance than reported by ROBINSON *et al.* (1955) for a series of quantitative characters in open-pollinated varieties of corn. As in that case, the findings are compatible with presence of partial or complete dominance at most loci. However, arguments concerning frequencies of favorable genes and genotype frequencies in the parental stocks cannot be advanced with the assurance that was possible in considering the corn data; and hence the evidence relative to the predominating levels of dominance is more ambiguous in this instance.

SUMMARY

Attention is drawn to a proposal by WRIGHT (1956) concerning the genetic situation to be anticipated in a species that is ordinarily asexual but in which biparental reproduction occasionally occurs. His proposal is interpreted to imply that, in a population (of such a species) derived entirely from biparental matings, there will be a large amount of total genetic variance and that a significant fraction of this variance will be of the epistatic sort. Evidence from an experiment with strawberries appears to conform with this interpretation of WRIGHT's comments.

Estimates of genetic variances have been reported for five quantitative characters in a population produced by random biparental matings among selected clones. Total genetic variance, evaluated in terms of the genetic coefficient of variation, was large for all five characters. Estimates of genetic variance among individuals within full sib families were too large to be rationalized in terms of associated estimates of additive genetic and dominance variance and were interpreted as due to presence of considerable amounts of epistatic variance.

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DELAY IN THE APPEARANCE OF CAFFEINE-INDUCED T5 RESISTANCE IN ESCHERICHIA COLI[1]

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IT is well known that the application of a mutagen usually does not produce the mutant phenotype immediately. A delay may be inevitable, for the expression of a genic change should require metabolic changes in the cell which cannot occur instantaneously, and which in turn may await other processes such as nuclear segregation. Indeed, if a variant phenotype were to be shown to follow treatment immediately, a more probable interpretation according to the present-day viewpoint would be that the variant arose through a cytoplasmic change.

Most studies of delayed expression have been made with bacteria, mainly with *Escherichia coli*. Some of the factors which must be considered for their possible contribution to delay have been listed by WITKIN (1956): the delay could occur in (a) *gene mutation* itself, (b) *nuclear*, or *genic*, *segregation*, (c) *phenotypic expression* due to prerequisite metabolic activity, (d) *irregularity of the growth pattern* when the mutagen interferes with the division rate of cells in the culture, and (e) *irregularity in the onset of division* of the mutant cells. All of these have been invoked as probable sources of delay in one previous study or another.

Induced delays range from about one to three divisions for streptomycin resistance (NEWCOMB 1953) to some 12 divisions for the appearance of the mutant phenotype of phage resistance (DEMEREC and LATARJET 1946; WITKIN 1953) and for an auxotrophic back mutation (Demerec and Cahn 1953). Furthermore, widely different delays have been observed in the expression of a given phenotype depending upon variation in culture conditions (NOVICK 1956; LABRUM 1953) and upon dose of mutagen (RYAN 1954). NOVICK observed a delay with the chemostat in the appearance of T5 resistance which depended upon the growth rate of the culture. LABRUM found that full expression of T5 resistance had a delay varying from about three generations in a chemostat to about 12 generations upon nutrient agar plates. RYAN inferred from studies of back mutation to histidine independence that the factor of phenotypic expression in the delay was less than one generation when induced by small exposures to ultraviolet light, but that large exposures could cause delays greater than 30 generations. He suggested that the long delays were caused by the delayed onset in division due to injury. Furthermore, NEWCOMB (1953) suggested that the relatively long delays observed in the expression of phage resistance were due to a selection artifact in the plate culture method.

In view of the unusually long delays obtained for phage resistance with the

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plate culture method, as well as variations occurring in this and other systems with change in culture conditions and mutagen, it might be supposed that there can be no significant measure of delay. Nevertheless, it is possible to assume that different experimental methods should reveal the same factors contributing to the delay despite different growth conditions, and, indeed, under similar growth conditions and at low doses of mutagen these factors should contribute to the same extent.

Conceptually, it is convenient to group the factors contributing to the delayed appearance of the mutant phenotype in the following manner. After the mutagen is applied to a cell, the *initial* component of the delay is that time required to alter the state of the genetic apparatus so that the mutation of the gene occurs, or will occur without further intervention by the mutagen. Once in this state, *primary* genetic and physiological factors can add their contributions to the delay. If large doses of the mutagen are used, it is reasonable to expect a change in the delay period due to concomitant physiological effects of the mutagen upon the cells. This change, which to our knowledge has only been observed as an extension of the delay (RYAN), may arise because of direct physiological injury to the cell by the mutagen, as well as through secondary physiological effects generated by cell products and cell debris not present at low doses of the mutagen. At vanishingly small doses of mutagen the primary factors are expected to give a constant contribution to the delay regardless of the mutagen used.

With this viewpoint, although delay may have a complex basis in any particular kind of experiment, measurement of the primary component would be expected to give the same result under similar growth conditions. If the time taken to establish the mutated gene following the addition of mutagen in low dose is variable but relatively short compared to the total delay, then the delays measured by different kinds of experiments should give similar values. Thus induced delays would be expected to give values similar to those for delays in which the genetic change is brought in by transduction or recombination.

In order to obtain the primary component of the delay it is probably necessary to observe additional experimental restrictions. Transferring cells to an alternate growth medium may give a different delay, especially if the average cell size changes. We find, for example, that the average cell volume is increased about fivefold when *E. coli*, B or B/1,t is transferred from the steady growth state in a lactate medium to the steady growth state in nutrient broth. For the same reason it is advisable to maintain a culture within a constant growth phase during a given experiment.

The use of the chemostat, or another method of continuous culture, has several distinct advantages over standard plate culture methods for the measurement of delay periods. Bacteria are in a steady growth phase under constant environmental conditions, except for a small perturbation upon addition of the mutagenic agent. Samples of the culture withdrawn from the chemostat have a constant concentration, so that in studies of phage resistance it is easy to maintain a fairly uniform multiplicity of phage to bacteria. Again, the bacteria are dispersed in

liquid medium which prevents shielding of a part of the population from the phage, as occurs when phage is sprayed on culture plates containing microcolonies. Thus the time of adsorption of the phage is more precisely determined in liquid media. Finally, in the subsequent growth of the phage-bacterial samples upon plates, only a small fraction of sensitive bacteria can survive infection, and these only for a short period of time. Since this fraction is constant, it does not affect the measurement of mutation rates or delay.

We report here measurements with the modified chemostat (KUBITSCHEK 1954) of the delay in the appearance of resistance to the bacteriophage T5 by *E. coli*, strain B/1,t. We find a mean delay of nearly 3.4 generations, which agrees well with the measurement of HAYES (1957), who observed that the full expression of resistance in recombinants required some four generations of growth of the recombinant genotype. In addition, we find the delay appears independent of the growth rate of the culture, the concentration of the mutagen, caffeine, and is the same at 26°C and 37°C. In these experiments we used tryptophan to limit the growth rate of the cultures. We are indebted to DR. A. NOVICK for the parent culture.

During the course of the experiments described in this paper we found two forms of the phenotype B/1.t/5 (resistant to the bacteriophages T1 and T5 and requiring tryptophan). The first of these, designated strain E (early), has a growth rate on nutrient agar which appears independent of the presence of T5 up to phage concentrations as great as $10^{11} ml^{-1}$. The other, strain L (late), has a growth rate greatly reduced upon nutrient agar in the presence of large concentrations of T5. E strain bacteria grow to countable colonies within 16 hours at 37°C in the presence of 10^9 T5 particles ml^{-1}, but the L strain colonies are smaller and cannot be counted reproducibly at this time. We customarily counted L strain colonies during the second day of incubation since the count remained stationary from that time on. In the absence of T5 the growth rates of the two strains appear equal. These characteristics of the two strains are reproduced in their progeny. The L variant had been found previously by A. NOVICK (personal communication). In addition, he found that the effect of T5 upon the delay in the appearance of L strain colonies essentially could be nullified by the addition of iron to the nutrient agar.

We observe both strains to accumulate linearly in the chemostat before as well as after the addition of caffeine as a mutagen. Thus, in the chemostat both strains have growth rates equivalent to that of the parent culture. However, strain E predominates until the addition of caffeine to the growth tube, after which the L strain accumulates more rapidly. We have measured the mean delay in the appearance of resistance to the bacteriophage T5 for both of these strains.

Theory

In steady state operation, mutants accumulate linearly in the growth tube of a chemostat when their growth rate is equivalent to that of the parent strain (NOVICK and SZILARD 1950) provided the mutant population is but a small frac-

tion of the culture. The application of a mutagen to the culture increases the rate of appearance of mutants after a time lag (NOVICK and SZILARD 1952). Examples of this delay period are shown in Figures 5 and 6 where the initial linear portion of each curve is extended to intersect the asymptote to the final portion.

We shall now show that this intersection occurs at that time following application of the mutagen which is equivalent to the mean delay period required for the appearance of the mutant phenotype. Let $p(t)$ represent the distribution function giving the probability for the appearance of the mutant phenotype at a time t following the mutational stimulus. The mutational stimulus is considered to be that action of the mutagen upon the cell which initiates the chain of events leading to the mutant genotype. For the moment we neglect the spontaneous background mutation rate. We assume that in the presence of the mutagen, which is added to the culture at time zero, the mutational stimulus has a small probability μ of occurring in any cell in unit time. Then the differential number of mutants induced between times ϕ and $\phi + d\phi$ for which the mutant phenotype appears between times θ and $\theta + d\theta$ is given by

$$d^2m = \mu N\, p(\theta - \phi)\, d\phi\, d\theta\,, \tag{1}$$

provided $m << N$, where N is the bacterial concentration, and m is the concentration of mutant bacteria. However, the test for the presence of these mutants will usually occur at some later time t, when the concentration of mutants in the population will then be

$$m = \mu N \int_0^t d\phi \int_0^t p(\theta - \phi)\, d\theta \tag{2}$$

It is convenient to make the transformations $\alpha \equiv t - \phi$ and $\beta \equiv \theta - \phi$. Then we can rewrite Equation 2 as

$$m = \mu N \int_0^t d\alpha \int_0^\alpha p(\beta)\, d\beta\,; \tag{3}$$

and integration by parts gives

$$m = \mu N t \int_0^t p(\beta)\, d\beta - \mu N \int_0^t \beta\, p(\beta)\, d\beta\,. \tag{4}$$

For times greater than that required for the expression of, say, 95 percent of the mutants, for which it may be assumed that the probability distribution function $p(t)$ either vanishes or decreases more rapidly than t^{-2}, the number of mutants approaches the asymptotic value

$$m_a = \mu N t \int_0^\infty p(\beta) d\beta - \mu N \int_0^\infty \beta\, p(\beta)\, d\beta\,. \tag{5}$$

The intersection of this asymptote with the abscissa, $m_a = 0$, gives

$$t_a = \frac{\int_0^\infty \beta\, p(\beta)\, d\beta}{\int_0^\infty p(\beta)\, d\beta}\,. \tag{6}$$

But formally, Equation 6 is also the definition of the mean delay in the appearance of the mutant phenotype.

When the spontaneous mutation rate, μ_o, is considered, then the number of mutants before the application of the mutagen is

$$m = m_o + \mu_o N t \,, \tag{7}$$

where m_o is a constant. The asymptote to the curve after the mutagen has been added is

$$m_a = m_o + \mu_o N t + \mu N t \int_o^\infty p(\beta) d\beta - \mu N \int_o^\infty \beta\, p(\beta)\, d\beta \tag{8}$$

Again, these intersect at the mean delay time.

MATERIALS AND METHODS

All measurements were made with cultures of strain B/1,t in the chemostat growing in Friedlein medium with tryptophan added in growth-rate-limiting amount (DL-tryptophan 100 or 1000 γ/l). In any given experiment, growth rate, temperature, and medium (except for the addition of caffeine) were not disturbed, although these and other parameters were varied between experiments. Caffeine was added both to the culture vessel and nutrient solution, usually at 450 mg/l, after the steady growth state had been reached.

Unless otherwise indicated, the parent population and the T5-resistant fraction were determined by standard methods described by ADAMS (1950). In testing for resistant bacteria the phage-bacterial mixtures were maintained for five minutes at 45°C before plating in an attempt to obtain phage adsorption upon all sensitive cells before pouring and solidification of the agar. There was no detectible decrease in the viability of parental or T5-resistant cultures in nutrient agar in the sol state at 45°C within the first ten minutes. In these mixtures phage concentrations were in the range 4×10^9 to 10^{10} ml^{-1}.

The phage-bacterial mixtures were often plated both upon Difco nutrient agar supplemented with 3 gm/l yeast extract and upon this same medium additionally supplemented with $FeCl_3.6\ H_2O$ at 100 mg/l. The agar layer technique with an additional sterile layer poured over the culture layer was used in determining the number of cells, primarily to increase the distinguishability between E and L strain colonies. E strain counts upon nutrient agar were made the day after plating. These plates were recounted the following day to determine total resistant cells (E + L strains). The addition of iron to the nutrient agar gave almost the same counts (E + L) on both days.

In order to compute the mean delay, the data from a given experiment must be fitted with two straight lines (as shown, for example, in Figure 5). The initial linear response and the asymptote to the final portion of the curve were fitted to the data by least mean squares. A mutation rate of 1.42×10^{-8} hr^{-1} bact^{-1} was assumed for the initial linear response. This value, with a standard error of 0.045 mutants hr^{-1} bact^{-1}, represents the average of six measurements of the spontaneous mutation rate. The slope of the asymptote was not assumed.

Following the introduction of the mutagen, a major difficulty in fitting the data is that of determining that initial point of the curve which may be considered to be indistinguishable from the asymptote to the tail of the curve. This difficulty

arises from the errors involved in determining bacterial concentrations by plating, and is in turn the major source of some of the larger errors estimated in Figure 1. In further discussion, it will be referred to as the "initial point difficulty".

RESULTS AND DISCUSSION

Figure 1 indicates the mean delay $\overline{D}$ observed for the E strain with chemostats operated under standard conditions: 37°C, caffeine added to a concentration of 450 mg/l, and the parent culture at 2 to 3×10^8 ml^{-1}. The variations shown are graphical estimates of the error of the individual delays determined in the following manner. For each experiment of the kind shown in Figure 5, the initial linear portion of the curve is replaced by a new line of reduced slope and reduced mean. The slope is reduced by the slope standard error, 0.045×10^{-8} mutants hr^{-1} $bact^{-1}$. The mean is reduced by the standard error of the mean calculated from the data.

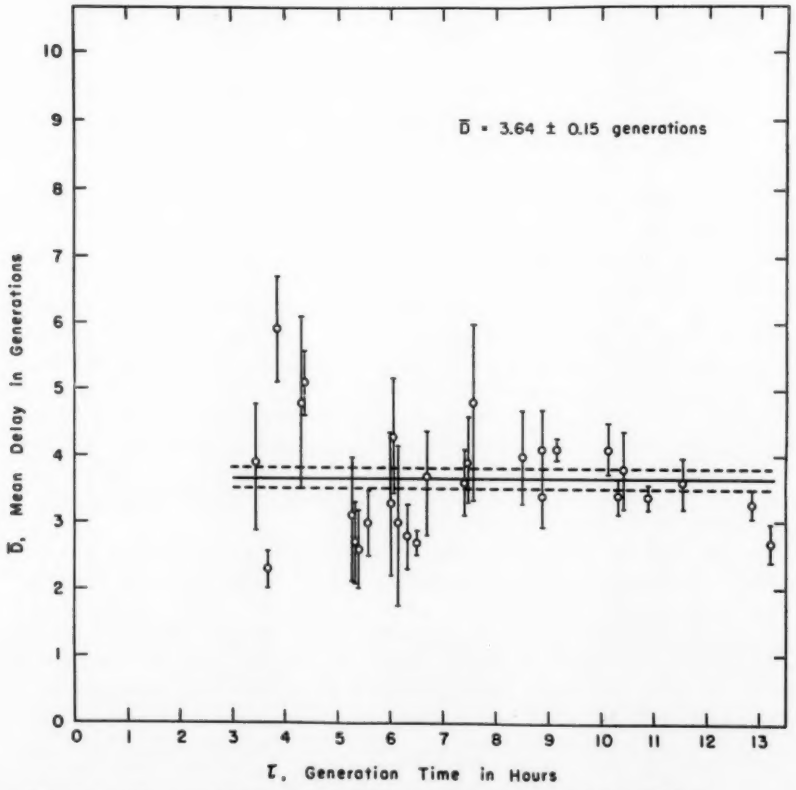

FIGURE 1.—The mean delays for the E strain observed under standard conditions.

Next the asymptote is replaced by a new line with reduced slope and reduced mean, but in this case the slope standard error is computed from the data. The intercept of these new lines gives a time shorter than the observed delay by approximately its standard error. In the absence of initial point difficulty preliminary calculations indicate that this estimate of the standard error is valid within 10 percent for our experiments. The standard error was also estimated in the same fashion by increasing the mean and the slope of both lines.

The data of Figure 1 are consistent with a constant delay, in terms of the number of generations (elapsed time/generation time) required for the expression of resistance to T5, for a generation time τ greater than three hours. One generation time is the time required to wash out a culture volume equivalent to that of the growth tube (NOVICK and SZILARD 1950); it is also the time required for the number of fissions which have occurred within the growth tube to be equivalent to the number of bacteria. Since a weighted average would contain the initial point difficulty, and thereby weight some data unduly, we present the unweighted average for the mean delay period for these data, $\bar{D} = 3.64 \pm 0.15$ generations for $\tau > 3$ hours.

An apparent increase in the delay was observed at generation times less than three hours. This increased delay is associated with the formation of filamentous bacteria at these more rapid growth rates. The filaments are readily demonstrated by microscopic observation, as well as by an apparent drop in plate count, but not in turbidity. Figure 2 indicates the plate count as a function of generation time in the presence of 450 mg of caffeine per liter. The formation of filaments also occurs at rapid growth rates in the absence of caffeine, but only rarely at generation times greater than 2½ hours.

Our interpretation of the increased delay is that T5 resistance is recessive to sensitivity, that segregation of T5 resistance is not possible in a nondividing filament, and that the development of the new phenotype must await the formation of more or less normal (homokaryotic?) cells by the reduction of the filament to cells of customary size. The hypothesis that T5 resistance is recessive is consistent with the observation that T1 resistance is recessive (LEDERBERG 1949). If, instead, T5 resistance were dominant, the culture fraction of mutant bacteria would appear to be unduly great in the presence of filaments and the mean delay period would appear shortened at rapid growth rates.

Since the formation of filaments increases abruptly at generation times less than three hours, we consider only those data for generation times greater than three hours.

It was realized that T5 concentrations which are too low should give too small a value for the mean delay: some of the cells with latent (cryptic) resistance will not be exposed to T5 before resistance is expressed, and the measured level of T5-resistant cells will be falsely high. To test that our data were not subject to this error three different concentrations of phage were applied to successive samples from a single chemostat containing caffeine. The results, shown in Figure 3, indicate that the phage concentration of 4×10^9 ml^{-1} was as effective as the higher

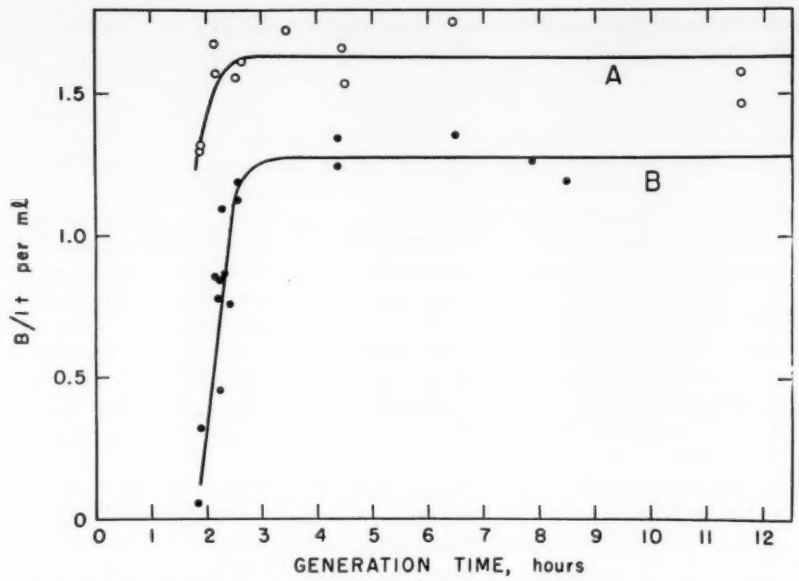

FIGURE 2.—The decrease in bacterial concentration, as determined by plate count, at rapid growth rates in the chemostat with a caffeine concentration of 450 mg/l.

A: limited with about 500 γ of L-tryptophan per liter.
Multiply ordinate by 10^8.

B: limited with about 50 γ L-tryptophan per liter.
Multiply ordinate by 10^7.

concentration of 1.5×10^{10} ml^{-1}; however, the ten-fold dilution to 4×10^8 ml^{-1} gave a delay period which was obviously shorter. The corresponding multiplicities of infection were estimated to be about 5, 30, and 0.5 phage adsorbed per bacterium, respectively. The two-day plate counts, which are not shown, gave parallel results. These experiments indicated that the phage concentration customarily used during adsorption, 4×10^9 ml^{-1}, was sufficiently high so that, within the accuracy of measurement, all cells with latent resistance were infected before division could ensue.

When the mean delay was measured by including the L strain colonies in the plate count it again appeared constant for $\tau > 3$ hours and not significantly different from the previous value for $\bar{D}$. This is indicated in Table 1 where the mean delay periods for the E strain and the E + L strains may be compared. A further comparison was made with delays for the L strain alone, the points being determined by difference between the one- and two-day plate counts on nutrient agar; again the delay agrees with that of the E strain. The E and L strains are similar also in that both appear highly resistant to T5: we were not able to demonstrate adsorption of T5 onto heat-killed cells of either strain in nutrient broth over a

period of four hours. However, the inhibition of the development of L strain colonies by T5 indicates an interaction, the nature of which has not been ascertained.

Measurement of the two strains together to determine the delay provides an advantage over a similar measurement for E or L strain alone. This advantage is not so much the increased number of resistant cells available, since these often must be diluted to avoid overcrowding of colonies upon plates, but results primarily from the increased angle at which the asymptote rises, which reduces the dispersion Δ in a measurement of the delay. When Δ is very small compared to

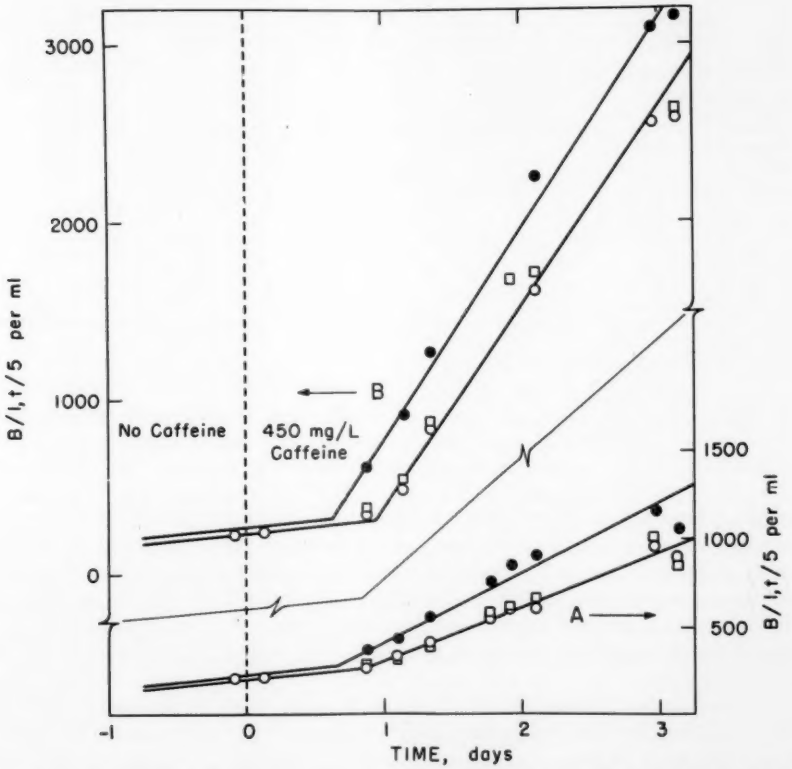

FIGURE 3.—The effect of several concentrations of T5. Data taken under standard conditions; B/1,t at 2.0×10^8 ml^{-1}, generation time of 7.5 hrs. The concentrations during adsorption were the following: ●, 4.3×10^8 phage ml^{-1}; ○, 4.3×10^9 phage ml^{-1}; for each, $0.6 - 2.9 \times 10^7$ bact ml^{-1}; □, 1.5×10^{10} phage ml^{-1} and $0.3 - 1.0 \times 10^8$ bact ml^{-1}. Caffeine added at time zero. A, from plate counts upon nutrient agar; B, from plate counts upon nutrient agar supplemented with ferric chloride.

TABLE 1

Measurements of mean delay periods

Experimental parameters varied	Range of generation times, hr.	Number of measurements	Mean delay, generations	Standard error, generations
All measurements under all conditions	3.2–16.6	70	3.42	.10
Measurements under standard conditions* only				
E strain on NA†	3.4–13.2	30	3.64	.15
E + L strain on NA	4.0–10.3	6	3.5	.2
E + L strain on Fe-NA‡				
Initial count	5.6–10.3	3	3.5	.0
Final count	5.6–10.3	3	3.0	.8
Standard conditions except for bacterial titer: $1–2 \times 10^7$ ml^{-1}	3.5, 11.3	8	3.1	.3
Measurements at 26° C. (titers: 2.0×10^8 ml^{-1}, 1.8×10^7 ml^{-1})	7.7–16.6	8	3.6	.3
All measurements of				
E strain	3.2–16.6	41	3.53	.14
E + L strain	3.5–16.6	29	3.26	.13
L strain	3.5–16.6	17	3.45	.18

* Standard conditions: caffeine at 450 mg/l, 37° C., and B/1,1 concentration $2–3 \times 10^8$ ml^{-1}.
† Nutrient agar.
‡ Nutrient agar supplemented with ferric chloride.

the time over which the final asymptote is measured, then it can be shown that

$$\frac{\Delta}{D} \sim \frac{1}{\sin A}$$

where A is the angle of intersection between the initial linear portion of the curve and the asymptote, and D is the mean delay for that measurement. At 37° C the caffeine-induced rate to T5 resistance for both strains is greater than twice that for the E strain alone. However, consideration of the final entries in Table 1 shows that the standard error was reduced very little. A greater reduction may have been prevented by the initial point difficulty, which contributes error to D asymmetrically.

The delay also is interpreted to be constant, in terms of generations of growth of the culture, for the following ranges of parameters: caffeine concentration, 75 to 450 mg/l (Figure 4); temperature, 37° C and 26° C (Table 1); bacterial concentration, 10^7 to 3×10^8 ml^{-1} (Table 1); and culture volume, 22 to 250 ml. Table 1 displays the mean delays and their standard errors under the assumption that each determination has the same validity. The constancy of the mean delay period at the two temperatures, 26° C and 37° C, agrees with the constancy, but not the extent of delay obtained for T1 resistance by WITKIN (1953). Measure-

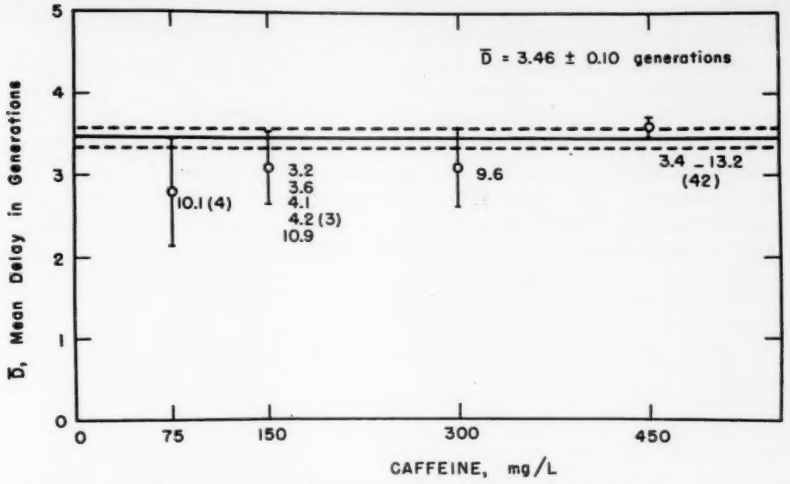

FIGURE 4.—The mean delay as a function of caffeine concentration at 37° C.

The generation times at which the delays were measured are indicated with the number of measurements at each time shown in parentheses when more than one measurement was made. Both E and E + L strain measurements were made. The solid horizontal line indicates the average 3.46 generations, of all measurements on the graph and the dotted lines the extent of the standard error, 0.10 generations.

ments with the chemostat have the advantage that growth rate and temperature can be uncoupled.

Figures 5 and 6 compare typical experiments at low bacterial concentration for 37° C and 26° C respectively, and also indicate the effect of iron upon the plate counts. At both temperatures the colony count increase occurring between the initial count (one day) and the final count (two days) is smaller upon medium supplemented with ferric chloride, because of the appearance of the L strain colonies in the initial count on iron-supplemented nutrient agar. It also may be noted that the spontaneous mutation rate is reduced from 1.4×10^{-8} bact^{-1} hr^{-1} at 37° C to about 0.5×10^{-8} bact^{-1} hr^{-1} at 26° C, while the relative reduction in the rate of mutation to the E strain in the presence of caffeine is even more marked, about 80 percent.

Figures 5 and 6 also show the advantage of reducing the bacterial density in the chemostat to about 10^7 ml^{-1}. Normally, with cultures of about 2×10^8 bact ml^{-1}, variants of greater growth rate appear within some 5 to 8 days, with the consequent displacement of the more slowly growing mutants from the chemostat (NOVICK and SZILARD 1950). This event terminates the experiment. But with reduced density, the parental strain B/1,t rarely is displaced within eight days by the more rapidly growing strain B/1,t/f, and displacement may be delayed for several weeks. Operationally, low bacterial concentrations permit a more precise

determination of mutation rates and mean delay periods with a single chemostat than would be possible with more dense cultures.

Finally, the data for all determinations averaged over unit intervals of generation time give the results shown in Figure 7. For generation times in excess of three hours the average of all mean delays (the E and E + L strain measurements), 3.42 ± 0.10 generations, can be seen to agree with the data, reinforcing the previous interpretation of a constant mean delay period. This result is at variance with Novick's study (1956), which gave delays of about two generations or less at comparable growth rates. (However, his measurements also included the use of antimutagens.) A shorter delay could be interpreted most simply as

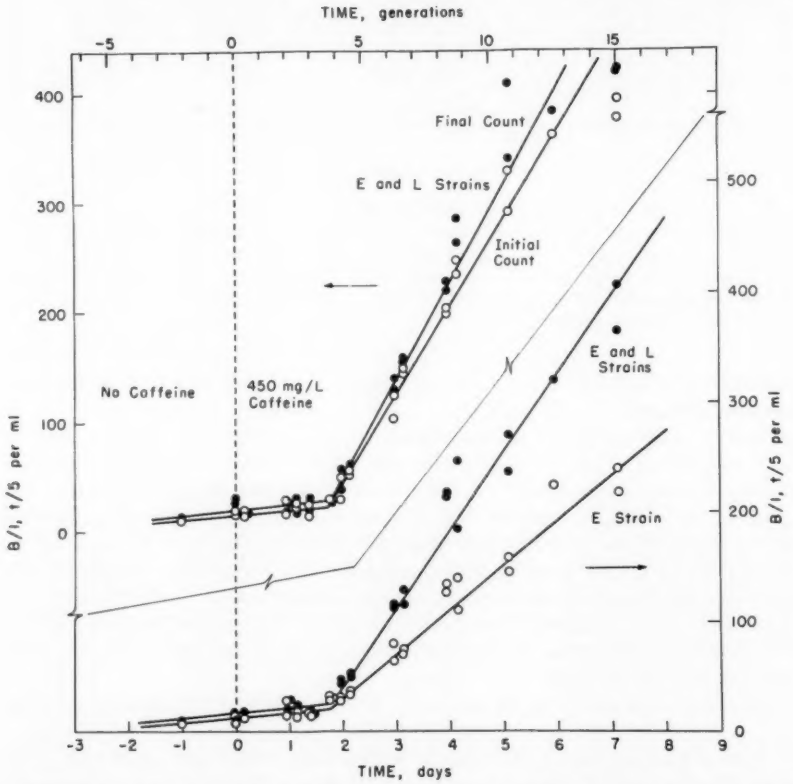

FIGURE 5.—The T5-resistant population in a chemostat with a bacterial concentration of 1.5×10^7 ml^{-1} at 37° C.

Generation time, 11.3 hr; caffeine, 450 mg/l. The two lower curves show the plate count increase between the initial and final counts on nutrient agar, the two upper curves for iron-supplemented agar. The lowest curve displays the appearance of the E strain only.

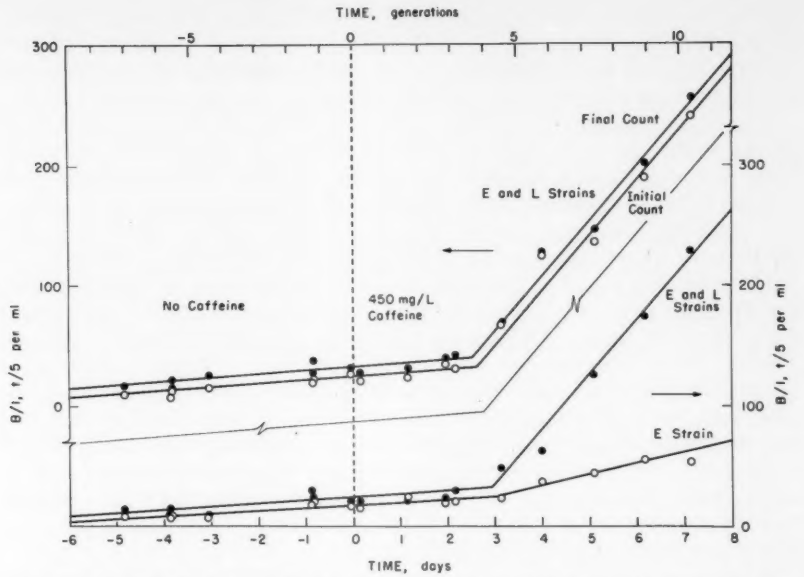

FIGURE 6.—The T5-resistant population in a chemostat with a bacterial concentration of 1.8×10^7 ml^{-1} at 26° C.

Generation time, 16.6 hrs; caffeine, 450 mg/l. The two lower curves show the plate count increase between the initial and final counts on nutrient agar, the two upper curves for iron-supplemented nutrient agar. The lowest curve displays the appearance of the E strain only.

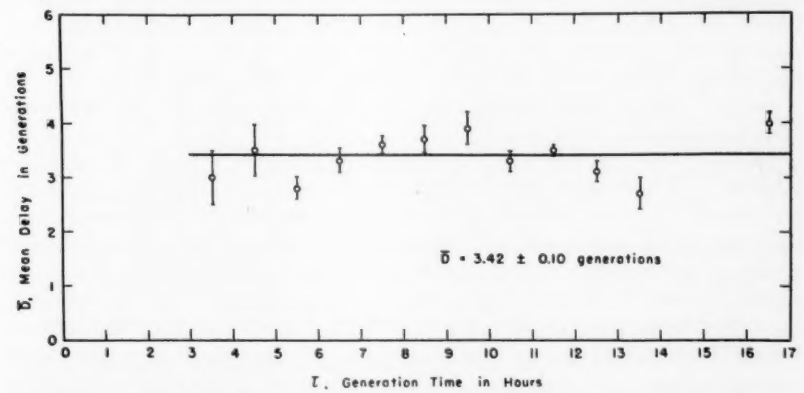

FIGURE 7.—The independence of the mean delay period with respect to generation time.

The data for all determinations have been averaged over unit intervals of generation time. The vertical bars indicate the extent of the standard error both above and below the average mean delay for the interval.

arising from the incomplete exposure of cells with latent resistance to bacteriophage. An alternative possibility is that the studies involve different strains of bacteria, but such an interpretation is not strongly suggested, since in both studies the spontaneous and caffeine-induced mutation rates are very similar.

The mean delay period of 3.42 generations is about half that usually observed for the mean delay to the phage resistant phenotype by the plate culture method. Thus these experiments also demonstrate that an artifact has been removed by using cultures in suspension at the time of application of the phage. This artifact was not present, of course, in the work of LABRUM (1953), who observed that the delay in T5 resistance induced with manganous chloride in a chemostat was not constant, but varied as the generation time was changed. As NOVICK (1955) has already pointed out, LABRUM's work is open to the difficulty that the bacteria were emerging from a long lag period, during which the mutagen had been applied, and the question of the irregularity in the onset of division now becomes predominant. However, our mean delay period can be seen to be in fair agreement with the delay measured for the expression of T1 resistance introduced by recombination (HAYES 1957), where full expression of resistance is delayed until the fourth recombinant generation. A more precise comparison of the two methods awaits the determination of the initial component of the induced delay and the number of divisions required after formation of the zygote to establish the initiation of the first division of the recombinant cells.

At this point it is tempting and customary, to speculate upon the immediate mechanisms controlling the delay period. Such speculation here would be premature, since our measurements of the delay have not established the delay in divisions of the newly-forming mutant, but have only measured the delay in terms of the average number of divisions undergone by the parent culture. Although the growth rate of the established T5-resistant population is equal to that of the parent culture, it cannot be expected *a priori*, that the growth rate of the fraction with latent resistance is also the same. At present the constancy of the delay, in terms of generations of growth, is as little understood as the constancy, in terms of chronological time, of the mutation rate in the same bacterium (NOVICK and SZILARD 1950).

However, the interpretation of a delay constant under a variety of conditions requires additional explanation beyond the allocation of time spent by the cell in, for example, nuclear segregation. In our estimation this constancy may imply that tryptophan limits the rate of transformation of the mutant to the same extent that it limits the growth rate of the cell. That is, just as tryptophan is required for bacterial growth ending in division, so is there a requirement for tryptophan by the newly formed mutant in order that the new phenotype may be expressed. Furthermore, this constancy of the mean delay period also implies that despite varying culture conditions, there is little or no change in the number of generations that are required for the cell to pass through nuclear segregation, or through any other state preliminary to the establishment of the mutant phenotype, when tryptophan limits the growth of the culture.

SUMMARY

When caffeine is added to the medium of a continuous culture (chemostat) of *Escherichia coli*, strain B/1,t, the mean delay period for the appearance of bacteria resistant to the phage T5 is 3.42 ± 0.10 generation times of the parent culture. No difference in delay is observed for several caffeine concentrations, bacterial concentrations, growth rates at two temperatures, and for the appearance of two mutant strains of T5-resistant bacteria, designated E and L, and differing in their growth rates in the presence of large concentrations of T5. These results indicate that the use of the chemostat has eliminated a selection artifact commonly observed when phage is sprayed upon cultures of agar.

In addition, for the case (these experiments) where the mutant culture has the same growth rate as the parent culture, it is shown mathematically that the mean delay period is measured by the time given by the intersection of two straight lines fitted to the data.

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THE EFFECT OF PHOTOREACTIVATING LIGHT ON THE MUTATION RATE INDUCED IN DROSOPHILA BY TERTIARY-BUTYL HYDROPEROXIDE[1]

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THE effectiveness of long ultraviolet and visible light (photoreactivating light) in reducing the number of mutations produced by mutagenic ultraviolet, when the light is applied as a posttreatment, has been demonstrated in a number of microorganisms (NOVICK and SZILARD 1949; NEWCOMBE 1950; KIMBALL and GAITHER 1949; HAAS *et al.* 1950; GOODGAL 1950) and in Drosophila (MEYER 1951; ALTENBURG and ALTENBURG 1952). Moreover, HAAS *et al.* (1950) have found that irradiation of the culture medium with visible light, subsequent to its irradiation with mutagenic ultraviolet but prior to inoculation of the bacteria, reduced the mutation rate produced in the bacteria by the irradiated medium alone, a result which they suggest may be due to the destruction of unstable molecules, such as peroxides, by the visible light. It had been shown previously (WYSS *et al.* 1948) that culture medium irradiated with mutagenic ultraviolet and from which all the hydrogen peroxide had disappeared still retained part of its mutagenicity, most of which was due to the presence of organic peroxides which could be removed by catalase, leaving the medium nonmutagenic. Since a certain organic peroxide (tertiary-butyl hydroperoxide) has been shown to be mutagenic when applied to the polar caps (early germ track) of Drosophila eggs (L. ALTENBURG 1954), the effect of treatment of the polar caps with long ultraviolet and visible light (3000–4200A) following upon tertiary-butyl hydroperoxide treatment has been investigated. The posttreatment with photoreactivating light has been found to be much less effective, if not entirely ineffective, in lowering the mutation rate induced by pretreatment with tertitary-butyl hydroperoxide alone.

METHODS

The polar cap ends of one group of dechorionated fertilized eggs of *D. melanogaster* were exposed for 20 minutes to the vapor of a 0.5 M solution of tertiary-butyl hydroperoxide in di-butyl phthalate, after the manner described in a previous paper (L. ALTENBURG 1954). At the end of this time the eggs were removed from the chamber containing the vapor and the polar caps of one half of the eggs were irradiated for an additional 20 minutes at a distance of 50 cm with the light from a 100-watt General Electric CH-4 spotlight lamp, a large percent of the output of which was radiation ranging in wave length from 3000 to 4200Å (photo-

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reactivating light). The other one half of the eggs were not subjected to posttreatment after being exposed to the peroxide vapor but were placed in the same chamber while the posttreatment was in progress. These eggs were shielded from the long wave length ultraviolet and visible radiation; and after the posttreatment period, both sets of eggs were removed from the chamber and allowed to develop in darkness. The polar caps of a third set of eggs were exposed to the vapor of only the di-butyl phthalate (in which the peroxide used in the treated series was dissolved) for 20 minutes, then placed in the posttreatment chamber but shielded from the irradiation. All three sets of eggs were treated in each daily experiment and the experiments were repeated on successive days until a sufficient number of eggs was obtained.

After the eggs hatched, males carrying speck (*sp*) as a marker in one member of the second pair of autosomes and nonspeck (sp^+) in the homologous chromosomes were selected and tested for the presence of lethal mutations by the *sifter* technique (MULLER 1951). The standard errors of the mutation rates are here calculated in such a way as to take into account the increase in error caused by some of the mutants having had a common origin (MULLER 1952).

RESULTS

The mutation rate induced in the eggs by treatment with tertiary-butyl hydroperoxide alone for 20 minutes was 5.4 ± 1.6 percent (835 tested chromosomes from 41 treated males). If data from all previous experiments in which the pole cells were similarly exposed to the peroxide vapor are included, the mutation rate becomes 5.8 ± 1.3 percent (1,471 tested chromosomes from 89 treated males). The mutation rate induced in the eggs exposed to posttreatment by long wave length ultraviolet and visible light was 5.1 ± 1.7 percent (434 tested chromosomes from 34 males). The mutation rate recovered from eggs exposed to the vapor of di-butyl phthalate alone (the solvent used for the peroxide) was 0.3 ± 0.3 percent (358 tested chromosomes from 21 males).

DISCUSSION

The present study shows that pretreatment of Drosophila eggs with tertiary-butyl hydroperoxide results in a mutation rate which either cannot be lowered by subsequent irradiation with long wave length ultraviolet and visible light (3000–4200Å), or, at least, which cannot be lowered to an extent comparable to the lowering (by as much as 50 to 80 percent) of the mutation rate obtained when pretreatment consists of irradiation by mutagenic ultraviolet light (2537Å), using the same experimental techniques. This result indicates, as one possibility, that the photoreparative process acts before peroxide formation, or at least before the formation of peroxide of the type used. It is also possible that the mutagenic pathway early becomes branched, with photorepair affecting only one branch, or certain branches of it, and not the "peroxide" branch. This is indicated by the fact that a fraction of the mutation rate induced by ultraviolet light cannot be reduced by photoreparative light. There exists a possibility that there may have

been a slight lowering of about 0.3 percent in the mutation rate of the posttreated series in the present experiments (or 0.7 percent when all previous peroxide experiments are considered); but because of the large statistical error involved in the polar cap method of treatment, it would not be possible to demonstrate so small a difference.

SUMMARY

Posttreatment of germ cells of Drosophila at the polar cap stage with photoreactivating light does not significantly lower the mutation rate induced by previous treatment of the material with tertiary-butyl hydroperoxide.

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THE TIME OF X-RAY INDUCTION OF CROSSOVERS AND OF TRANSLOCATIONS IN DROSOPHILA MELANOGASTER MALES[1]

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WHEN crossovers are induced in *Drosophila melanogaster* males by X-ray treatment, the question arises as to whether they are more akin to spontaneous crossovers or to translocations. A spontaneous crossover, as it occurs in females, is defined as an equal exchange between homologous chromatids which presumably occurs during the first meiotic prophase. On the other hand, translocations, either spontaneous or induced, are usually considered to be the result of exchanges between nonhomologous chromatids; these exchanges are not necessarily equal. It has been demonstrated that X-ray treatment of adult flies will, after a time lapse, increase the frequency of recovered crossovers (MAVOR 1923) and of recovered translocations (MULLER and ALTENBURG 1930).

While studying X-ray effects on crossing over, MULLER (1925, 1926) noticed that the marked regions on the long autosomes showed different susceptibilities to treatment, i.e., the effect was concentrated in the spindle attachment region. MULLER also observed, upon comparing his results with those of MAVOR (MAVOR 1923, MAVOR and SVENSON 1924), that the heavier doses used by MAVOR produced a stronger effect in the central region than did the light doses used by MULLER. It was later found that X-rays would also induce crossovers in males as well as in females (FRIESEN 1933, 1934). FRIESEN observed that various genotypes responded unequally to induction of crossing over, but that when crossover offspring did occur, they carried cytologically normal chromosomes. The susceptible region was again the spindle attachment region, as had been shown earlier for females, and FRIESEN accepted this as an indication that the mechanism was identical in both sexes. FRIESEN's observations on regions of induction were confirmed by PATTERSON and SUCHE (1934).

The possibility that induced crossovers might occur in the gonial cell prior to meiosis was suggested by WHITTINGHILL (1937) on the basis of his study of crossovers induced by heat treatment of larval stages. This same suggestion had been made by FRIESEN (1936, 1937) on the basis of clustering of crossovers obtained from males ten or more days after treatment. FRIESEN felt that crossing over was an exchange of homologous loci which showed a specific attraction for each other. The occurrence of oögonial crossing over in females was demonstrated

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by WHITTINGHILL (1938) by X-irradiating females carrying the asynaptic factor *c3G*.

The relationship between dosage and frequency of induced crossovers might determine whether the two chromatid breaks necessary for a crossover were the result of a single ionization. PARKER (1948) contended that if the two breaks producing a crossover were the result of a single ionization, then the frequency-dose curve would be a straight line at low doses, and at higher doses would tend to level off as the upper limit of crossing over was reached. His results, based on the treatment of Drosophila males with four different doses of X-irradiation, seemed to follow this prediction.

Thus the evidence to date would indicate that X-ray induced crossing over in Drosophila males occurs primarily in gonial cells, but may occur occasionally during the first meiotic prophase. As to the relationship of induced crossover frequency to dose, no clear cut information has been obtained. Such evidence would allow a comparison with the better understood phenomenon of induced translocations (MULLER 1954), and thus possibly indicate the manner of induction of crossing over.

Several observations concerning factors which might influence or be influenced by the translocation frequency have been reported. No positive correlation between the region of breakage and lethals induced at the time of irradiation has been noted (PATTERSON, STONE, BEDICHEK, and SUCHE 1934). The induced translocation frequency between the II and III chromosomes was not affected by whether an irradiated sperm carried the X or the Y chromosome (SHAPIRO 1936). It has also been demonstrated that cold applied to mature sperm during irradiation increased the frequency of translocations produced by X-rays (MICKEY 1939).

On the basis of the work on induction of crossovers and translocations by X-ray treatment mentioned above, further information concerning the induction of crossing over could be obtained by X-irradiating adult males of *Drosophila melanogaster* at varying doses, and testing them for the frequency of induced crossovers and translocations. Spontaneous crossing over is known to occur rarely if at all in male Drosophila (MORGAN 1912, 1914; MULLER 1916; BRIDGES and MORGAN 1919), and thus any crossovers recovered in the experiments described in this paper probably would be radiation induced. By means of a series of successive matings over a 12 day period, a comparison between the time of recovery of induced crossovers and the time of recovery of induced translocations is made. In this manner information might be obtained as to which stages of spermatogenesis are most likely to be induced to form translocations and which to form crossovers. Also, information might be obtained as to the dependence on one or more ionizations of the two chromatid breaks involved in a crossover.

MATERIAL AND METHODS

Two *Drosophila melanogaster* stocks containing both second and third chromosome mutants were used for these experiments. One carried the second chromosome dominant mutants Jammed (*J*) and Blackoid (*Bkd*), and the third chromo-

some mutant Prickly (*Pr*). The second was a homozygous stock showing the recessive mutants light (*lt*), straw (*stw*), and plexus (*px*) of the second chromosome, and the mutants thread (*th*), scarlet (*st*), and stripe (*sr*) of the third chromosome (Figure 1). Thus the spindle attachment regions for chromosomes II and III were marked by mutants for a short distance to the left, and each right arm was marked for almost its entire length. This permitted observation of induced crossing over in the spindle region and in the distal region of the right arm, and allowed a comparison between the frequencies in the different parts of each chromosome. Translocations between chromosomes II and III as well as those involving II and III with either the X or Y chromosomes could be genetically detected.

The males used in these experiments were treated with X-irradiation by means of a Picker X-ray machine operating at 140 kv and 20 ma with filters of 1/4 mm Copper and 1 mm Aluminum. The dose rate was 163.93r/minute at 18 cm distance. The males to be treated in these experiments were from three to ten hours old at the time of irradiation. These males were placed in gelatin capsules and confined at one end of the capsule by a fine mesh gauze. The end containing the flies was punctured with five holes to provide ventilation. From five to seven males were placed in each of the capsules used, including one control capsule. Five experiments were conducted, each with five or six groups of males receiving different doses of X-irradiation. The capsules containing flies to be irradiated were mounted on Scotch tape across the opening of the aperture plate, and this, in turn, was attached to the X-ray machine. Doses in 500r steps from 0 to 4500r were used (Table 1). Single capsules were removed from the aperture plate at specified intervals and marked with the amount of radiation delivered at that time.

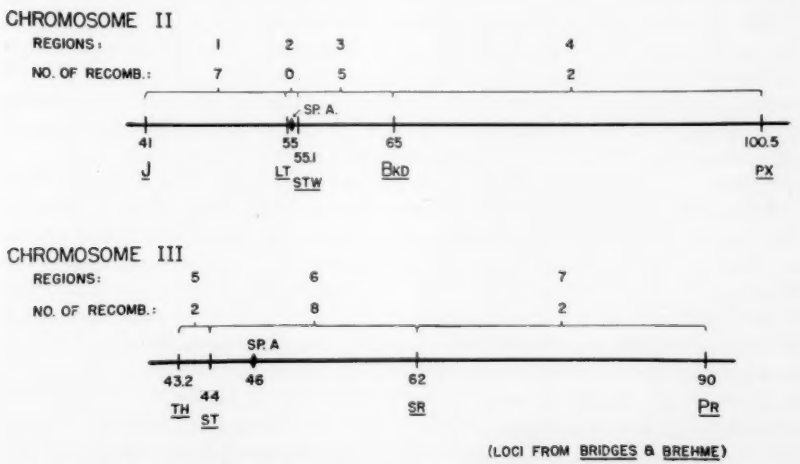

FIGURE 1.—Number of recombinations from males by designated regions.

TABLE 1

Number of males irradiated in each dosage group, by experiment and date

Dose	Experiment number and date irradiated					Total
	1 5/23/55	2 6/24/55	3 1/20/56	4 2/16/56	5 3/15/56	
Control	1	.	5	5	5	16
500r	4	5	.	.	5	14
1000r	4	4	4	5	.	17
1500r	3	5	5	.	.	13
2000r	4	5	.	5	3	17
2500r	.	5	5	.	.	10
3000r	.	.	5	4	4	13
3500r	.	.	4	.	.	4
4000r	.	.	.	4	5	9
4500r	.	.	.	5	5	10
						123

The breeding procedure for crossover detection covered two generations. In order to obtain males heterozygous for all nine mutants, *J Bkd; Pr* males were mated to virgin homozygous *lt stw px; th st sr* females, and males which showed the mutants *J Bkd; Pr* were selected from among the offspring. The heterozygous males carried *J Bkd* in repulsion phase to *lt stw px* in the second chromosome, and *Pr* was in repulsion phase to *th st sr* in the third chromosome. Shortly after these heterozygous males were irradiated in the manner described above, each male was mated to three virgins of the homozygous *lt stw px; th st sr* type. The males were then transferred to fresh vials and remated to three multiple recessive virgins on the third, sixth, and ninth postirradiation days. The males were destroyed on the twelfth postirradiation day. If the male was sterile or died before the series of four matings was completed, his whole series was discarded. The females were allowed to remain in the vials a total of six days before being removed. Each of these cultures (TC_1) from the irradiated males was classified for crossovers on the twelfth and sixteenth days after mating.

To investigate induced translocations one additional generation was bred. New matings were made from the TC_1 cultures on the twelfth and sixteenth days by selecting two males of *J Bkd; Pr* phenotype, which were, therefore, still heterozygous for all nine markers. The selection procedure consisted of arranging males of the proper genotype in a linear order as if numbered, and then selecting those to be tested for the presence of translocations by means of a random numbers table (FISHER and YATES page 107, 1948). These males were mated to three homozygous *lt stw px; th st sr* virgins and allowed to remain in the culture six days before being discarded. On the fourteenth day the TC_2 flies from the irradiated males were classified as to the presence of males and females in each of the four expected classes. If only P_1 classes appeared, then a translocation between the II and III chromosomes was assumed. Should one of the sex chromosomes be involved in a

translocation with either the II or III, then four classes would result with all the members of a class being of one sex.

Confirmatory tests by breeding from each suspected crossover fly and from TC_2 families which showed translocations were performed.

RESULTS

The offspring to be classified in TC_1 and TC_2 were recovered in four successive cultures or broods, which may be related to the stage of spermatogenesis in which the sperm utilized in that brood were irradiated. In the mating procedure mentioned above the irradiated F_1 males were crossed to three virgins following irradiation; this was designated the First Brood. These males were then remated in fresh cultures on the third, sixth, and ninth days following irradiation. The subcultures were designated as Second Brood, Third Brood, and Fourth Brood respectively. An approximate correspondence between the stages of spermatogenesis and the four cultures in which each male was successively bred, is presented below.

1st Brood—Days 1 to 3 postirradiation; Stage at time of treatment: mature sperm and spermiogenesis

2nd Brood—Days 3 to 6 postirradiation; Stage at time of treatment: second meiotic division and early spermiogenesis

3rd Brood—Days 6 to 9 postirradiation; Stage at time of treatment: first meiotic division, and probably a few early second meiotic stages and late gonial cells

4th Brood—Days 9 to 12 postirradiation; Stage at time of treatment: spermatogonial cells and early first meiotic cells.

This assignment of stages of spermatogenesis to utilization in specific broods was a provisional one, based on the work of AUERBACH (1954) and COOPER (1950).

The distribution of the number of males irradiated at each dosage level in each experiment is seen in Table 1. A total of 123 F_1 males were tested in the manner described: 16 controls and 107 X-irradiated experimentals.

The induced crossovers recovered from classifying the TC_1 offspring of the irradiated males are summarized in Table 2. The ten different doses given to the males throughout all the experiments are indicated in the first column and will be referred to as dosage groups. The crossovers are recorded not only in respect to the dosage the F_1 male received but also as to the brood from which they were recovered. No crossovers were recovered from the First Brood. Only two crossovers were recovered in the Second Brood, of which one was confirmed by breeding again, and the other (in Table 2) unconfirmed. Five crossovers appeared in the Third Brood. A majority of the confirmed crossovers, 13, were found in the Fourth Brood. These 13 included five double crossovers and one triple crossover. There were also three unconfirmed crossovers found in this brood. Those crossovers marked by an asterisk (*) were ones which were phenotypically classified as crossovers but which could not be verified by further testing, either because of sterility or death. Crossovers marked thus will not be considered in further dis-

TABLE 2

Crossovers recovered by brood and dosage group

Dose	1st brood	2nd brood	3rd brood	4th brood	Sperm tested	Crossovers recovered	Percent verified crossovers
Control	....	....	....	.....	3800	0	0.0±0.043
500r	....	....	*J lt stw Bkd; th st sr* ♀ **J lt stw Bkd; th st sr* ♀	*J lt stw Bkd; Pr* ♂ *J Bkd; th st Pr* ♀ **J lt stw Bkd; th st sr* ♀	2953	3(+2*)	0.10±0.058
1000r	....	....	....	*lt stw px; sr Pr* ♀ *+ px; Pr* ♀	3948	2	0.05±0.036
1500r	....	....	....	*+ px; th st sr* ♂ *lt stw px; th st Pr* ♂	2780	2	0.07±0.050
2000r	....	....	....	*lt stw px; th st Pr* ♂ *lt stw px; sr* ♂	3687	2	0.05±0.037
2500r	....	....	....	**J Bkd; th st sr Pr* ♀ *J Bkd; +* ♀	2334	1(+1*)	0.04±0.041
3000r	....	....	....	*J Bkd; sr* ♂ *+ Bkd; th st sr* ♂ **lt stw +; Pr* ♀	2774	2(+1*)	0.07±0.050
3500r	....	....	....	.....	760	0	0.0±0.096
4000r	....	*lt stw +; Pr* ♂ **J Bkd; st Pr* ♀	*lt stw px; th st Pr* ♂	.....	1567	2(+1*)	0.13±0.091
4500r	....	....	*+ Bkd; Pr* ♂ *J Bkd; st Pr* ♂	*+; Pr* ♀ *J lt stw Bkd; th st sr* ♂	1976	4	0.20±0.10
Sperm tested	7134	6918	6889	5648	26,589	.	.....
C.O. recovered	0	1(+1*)	4(+1*)	13(+3*)	....	18(+5*)	.....
Percent verified C.O.	0.0	0.014± 0.0141	0.058± 0.029	0.23±0.064	....	.	0.07±0.02

* Crossovers recovered but not verified by further testcrossing.

cussions unless specifically mentioned. They are indicated in parentheses in the column headed, "Crossovers Recovered," as additional crossovers occurring in that dosage group. Twenty-six thousand, five hundred and eighty-nine viable sperm were tested, and from these 18 crossovers were verified, thus giving an

over-all frequency of 0.07 ± 0.016 percent. If the unconfirmed crossovers were to be included in the total, the over-all frequency would be 0.09 ± 0.018 percent.

The X-ray induced translocations observed by genetic testing are recorded in Table 3. The translocations were sought by crossing TC_1 *J Bkd/lt stw px; Pr/th st sr* males to multiple recessive females, and observing the presence or absence of the eight equally expected classes in the TC_2.

Table 3 is arranged in the same manner as Table 2, with the dose appearing in the first column referring to that received by the F_1 male. Although translocations were detected in all four broods, the majority of them occurred in the Second and Third Broods. Approximately the same number of TC_2 cultures were made for each brood. This would suggest, in view of the relationship of the different broods to spermatogenesis, that translocations were induced most readily in cells undergoing meiosis at the time of irradiation. One thousand six hundred and fifty-five F_1 sperm were tested, and from these 20 translocations were recovered. This gave an over-all frequency for translocations of 1.2 ± 0.26 percent.

TABLE 3

Translocations recovered by brood and dosage group

Dose	1st brood	2nd brood	3rd brood	4th brood	Sperm tested	Total	Translocation frequency percent
Control	....	....	....	....	225	0	0.0±0.75
500r	....	....	....	....	160	0	0.0±0.86
1000r	....	....	....	....	219	0	0.0±0.74
1500r	....	II–III	II–III II–III	II–III	160	4	2.5±1.23
2000r	....	....	II–III II–III	....	225	2	0.89±0.63
2500r	....	....	II–III	....	144	1	0.69±0.69
3000r	II–III	Y–III	II–III II–III	II–III	198	5	2.5±1.11
3500r	....	....	....	....	61	0	0.0±1.39
4000r	....	II–III II–III Y–III	....	....	129	3	2.3±1.32
4500r	....	II–III II–III	II–III Y–III II–III	....	134	5	3.7±1.63
Sperm tested	442	424	381	408	1655	.	.....
Trans. recovered	1	7	10	2	...	20	.....
Percent trans. frequency	0.23±0.23	1.65±0.62	2.62±0.18	0.49±0.346	...	.	1.2±0.26

A small number of clusters involving crossovers and/or translocations from one irradiated male were encountered, and these were of the following types: (1) sibling crossovers—two cases, (2) sibling crossovers and translocations—three cases, and (3) sibling translocations—two cases. The 4500r dosage group showed still another type of clustering, of which only one example occurred. The Third Brood crossover *J Bkd; st Pr* male was also a II–III translocation recorded in Table 3. Moreover, this II–III translocation-crossover carrying fly was the sib of the Y–III translocation, found in the Third Brood. However, clustering did not appear to be excessive in any of these cases, as will be shown later.

When examining the frequency of crossovers for the various doses, it could be seen that while the frequency was increased in irradiated males, the increase did not appear to be a direct one as the total dose increased (Table 2). The control group, although it was the second largest group tested, showed no spontaneous crossovers. An unexpectedly high frequency of crossovers induced with the 500r X-ray dose was then observed. The frequency for doses from 1000r to 3000r was approximately the same but lower than the 500r group. The appearance of no induced crossovers at 3500r may be attributable to the very small sample tested. At 4000r, the frequency of crossovers was markedly high, and it was still higher at 4500r. The sampling error at each dose is, however, rather large. When the crossovers were distributed by brood, the highest frequency of crossing over was observed in the Fourth Brood.

The translocation frequencies with their standard errors are presented in Table 3. Below 1500r no translocations were recovered. As was seen in the crossover table the first dose at which any positive results were obtained showed a high frequency. There was a lowered frequency at 2000r and 2500r, and then a greater frequency at 3000r and 4000r. The 3500r group showed no translocations but this was again probably due to the small sample. The highest frequency obtained was in that dosage group receiving the largest total dose of radiation. Again the sampling errors for each of the dosage groups are large, due to the low frequency of recovered translocations. The results, when broken down into broods in which the translocations were recovered, showed the Second and Third Broods to have the highest incidences.

When the induced recombinations occurring in the treated males are classified by region of crossing over, as based on the female linkage map, a definite concentration of events can be seen (Figure 1). In all, there were 14 recombinations involving chromosome II, and 12 involving the marked regions of chromosome III. The majority of the recombinations involved regions near the spindle attachment, while only two were found in each of the distal regions, 4 and 7. This distribution of recombinations agreed with that reported by FRIESEN (1933); PATTERSON and SUCHE (1934); and WHITTINGHILL (1951).

DISCUSSION

In newly eclosed Drosophila male, there are present in the testis all stages of spermatogenesis from spermatogonial cells to mature sperm, and the process of

spermatogenesis continues for some time after eclosion, approximately 32 days (DUNCAN 1930). When young males are irradiated, it is assumed that all these stages receive the same treatment, the dose being uniform. If these males were then bred to a succession of virgins, the sperm utilized in the first mating would contain chromosomes treated either in mature sperm or in transforming spermatids. With each successive mating the male has available in the sperms, chromosomes which were treated at earlier and earlier stages in their development. A breakdown of stage of spermatogenesis at which treatment occurred, and the brood in which offspring resulting from those sperm might appear, has been proposed.

The time of appearance indicated that crossovers were formed primarily in the spermatogonial cells. Table 2 shows that one confirmed and one unconfirmed crossover appeared in the Second Brood, which was earlier than might be expected, and four confirmed crossovers were found in the Third Brood. The culture producing the majority of confirmed crossovers was the Fourth Brood, which resulted chiefly from irradiated gonial cells. A total of 13 crossover flies were recovered. These included five double crossovers, four of which could not have been the result of a deletion, and one triple crossover. Thus not only single crossovers, but double and triple crossovers were induced.

From the wide distribution of recovered translocations in the four broods, Table 3, it appeared that translocations could be induced at almost any stage of spermatogenesis. However, when these translocations were viewed as to the number of occurrences in the various broods, it was seen that 85 percent of the translocations were recovered in the Second and Third Broods, i.e., from cells irradiated during meiosis and early spermiogenesis. AUERBACH (1954) reported, incidental to a study of induced mutations and crossovers, that she had recovered all of ten translocations from the same three to nine day postirradiation period which produced the major portion of those found here.

While certain stages of spermatogenesis have been assigned to specific broods, this is not an absolute or inflexible scheme. The factors which would affect this scheme are several. The age differences between the irradiated males, which were from one to seven hours, individual and stock differences, internal and external environmental factors, and the number of virgins available for mating would all influence the rate of spermatogenesis. With these considerations in mind the scheme as proposed above can not be taken as rigid, and a certain amount of overlapping of recovered translocations and crossovers within the same brood should be expected.

It had been thought that the type and quantity of clustering observed in these experiments might give some information as to the stages of spermatogenesis most apt to allow reunion of chromosome breaks as crossovers or as translocations. From these clusters of crossovers and/or translocations it would appear that the tendency to form either crossovers or translocations from chromosome breaks might not be as restricted to specific stages as had been supposed. There was no basis for assuming that breaks in late gonial cells could not produce translocations,

or that crossovers could not occur in the early stages of meiosis, except that these phenomena were not usually found in broods supposedly resulting from sperm treated at those stages.

Possible direct evidence that one stage of spermatogenesis was capable of producing both a crossover and a translocation with no definite separation of these phenomena to specific stages of development might be found in the 4500r dosage group. It was in this group that there appeared an example of overlapping results within one individual and within one brood, i.e., the Third Brood double crossover *J Bkd; st Pr* which was also a II–III translocation, and had a sibling Y–III translocation. Yet, if the apparent double crossover were due to a deletion of the wild allele at the *st* locus, or if the translocation involved a break and consequent damage at that locus, this would be merely a II–III translocation with a sibling Y–III translocation. This single event might also be explained as a spontaneous II–III translocation occurring later than the induced crossover, or as a result of a delayed X-irradiation effect. While there was no direct evidence for any of these explanations, they were necessary considerations and should be taken into account where one such unusual coincidence has been observed.

When the observed frequency of these various types of clusters was compared to the expected frequency, which was based on the frequency of independent events for each phenomenon, there appeared to be no significant differences. Thus the possible overlapping of phenomena observed from one male, which might have indicated that both translocations and crossovers could be induced in the same stage of spermatogenesis, was no more than expected. The one case in which the same fly was both a crossover and a translocation and which might still be considered evidence for the induction of these two phenomena in the same stage of spermatogenesis, was also within the realm of expectancy even if it was not the result of a deletion.

If the pattern of sperm treated at various developmental stages and the utilization of those sperm for fertilization in the successive cultures which has been used here is correct, then some conclusions can be drawn from the distribution of crossovers and translocations in the four broods as seen in Tables 2 and 3. On the basis of recovered offspring it appeared that mature sperm were relatively refractory as regards induction of crossovers and translocations by X-irradiation. Viable translocations appeared to result from cells treated at any stage, while viable crossovers were recovered primarily from cells treated in the spermatogonial stage. Thus there seemed to be a specific stage of spermatogenesis at which reunion of the induced chromosome breaks resulted in crossing over, as judged by inference from the live offspring.

These experiments were designed to study not only the time of appearance of these phenomena, but the dosage relationship to the frequency of crossing over and to the translocation rate. The over-all production of crossovers and probably translocations has been shown to be related to the genotype of the stocks used (Friesen 1933). This procedure therefore allows for a simultaneous comparison

of the two rates, one of which has seldom been investigated before the present study.

The frequency of recovered crossovers and of translocations at each dosage level can be seen in Tables 2 and 3, and there is no evidence of the spontaneous occurrence of either phenomenon. Because of the large standard errors which must accompany each of these frequencies, it is difficult to make any definite statement as to the relationship between crossover or translocation frequencies and dose. Nevertheless, there is a suggestion that both frequencies increase with higher doses.

Should the crossover frequency have followed a linear relationship to the dose, as was the case in induced mutations, then the two homologous chromatid breaks necessary for the crossover to occur would probably have resulted from a single ionization or a small cluster of ionizations similar to those from the end of an electron track. Breakage in this manner would have insured equal crossing over and therefore viable zygotes. If, on the other hand, the two homologous chromatid breaks were the result of two independent ionizations, one would have expected a crossover frequency-dose curve similar to that for translocations. The two breaks would necessarily have had to occur at or very close to the same locus in order to have resulted in a viable crossover chromosome. Therefore, if breaks occurred more or less at random on the chromatids as was shown by KAUFMANN and DEMEREC (1937), the expected frequency of translocations would have been much greater than that of almost equal "two-hit crossovers" at any one dose level. The results of the present study tend to support this hypothesis.

Attention might now be directed to the relative differences in frequency of crossovers and translocations in each dosage group. At doses of 1500r and 3000r the frequency of translocations was approximately 35 times that of crossovers; while at 2000, 2500, 4000, and 4500r the difference was about 18 times that of crossovers. Because the frequencies of translocations and crossovers were based on very small numbers of events, the differences between these frequencies at the various dosage levels could shift from 35× to 18×, or vice versa, with the addition or subtraction of one or two events. The main point here was that the frequency of observed translocations was at least 18× the frequency of observed crossovers for the individual dosage levels, and for the over-all frequency. If crossing over were the result of two chromatid breaks which were dependent on the same ionization, then one would expect induced crossovers to be at least as frequent as translocations requiring independent breaks. On the basis of the high frequency of translocations recovered in the Third Brood it would seem that this was the brood or stage of spermatogenesis in which the number of single chromatid breaks was most numerous. When the number of recovered crossovers in the Third Brood is observed, it can be seen that the frequency is 1/45 that of recovered translocations. Thus it would appear that the single chromatid breaks occurring at this stage of spermatogenesis were unable to form viable crossovers. When the results of the Fourth Brood are viewed, the picture has changed. The frequency

of recovered and verified crossovers is at its highest point, 0.23 percent, while that of translocations is quite low in comparison with Broods Two and Three. However, the percentage of recovered translocations is approximately twice that of crossovers, seeming to indicate that while there are breaks occurring and forming translocations, a much larger percentage of these breaks is being utilized in crossover formation, if crossing over is a breakage phenomenon.

It would appear from the picture presented by the Third and Fourth Broods that the single chromatid breaks which occur in spermatogonial cells and very early first meiotic stages form crossovers in competition with translocations, while chromatid breaks occurring in cells in later stages of meiosis preferentially form translocations rather than crossovers. The overlap of observed results in these broods, and especially the Fourth Brood translocation frequency of almost double the crossover frequency, might be explained in two ways. As has been pointed out before in this paper, the division of irradiated stages of spermatogenesis into specific broods in which they might be recovered, while reasonable, was provisional to some extent in that too little is known about the rate of spermatogenesis and the utilization of sperm in fertilization. It is then quite possible that there would be some overlap in observed results, and with the small number of translocations and crossovers recovered in these experiments, very little overlap would result in frequency differences of 1× or 2×.

Following the premise that single chromatid breaks occuring in spermatogonial cells and early first meiotic stages preferentially form crossovers, the frequency of twice the number of translocations as crossovers in the Fourth Brood could be explained on the basis of duplications and deficiencies. Unequal "crossovers" resulting from single chromatid breaks not at or very near the same locus would result in duplications and deficiencies which could lead to an adverse selection against "crossovers" formed from such random single breaks; whereas translocations resulting from these breaks would be viable. Such selection against "crossovers" occurring from two random single chromatid breaks in the spermatogonial-early first meiotic stages might well explain the much lower frequency of crossovers as compared to translocations recovered in this study.

Induced crossing over in the long autosomes of either males or females has been shown to occur primarily in the central region, (MULLER 1926; FRIESEN 1933; PATTERSON and SUCHE 1934; WHITTINGHILL 1951). This fact was corroborated by the results of these experiments as they are presented in Figure 1.

SUMMARY

1. *Drosophila melanogaster* males carrying the mutants *J Bkd/lt stw px* in chromosome II and *Pr/th st sr* in chromosome III were treated with doses of X-irradiation ranging from 0–4500r. These males were then testcrossed to homozygous recessive virgins on days 0, 3, 6, and 9 postirradiation, and their offspring, TC_1, classified for induced crossovers.

2. TC_1 males carrying *J Bkd/lt stw px; Pr/th st sr* were randomly selected and

bred to recessive virgins. Their offspring, TC_2, were classified to detect induced translocations.

3. Eighteen verified crossovers comprised 0.07 ± 0.02 percent of the TC_1 generation. There were 11 single crossovers, three of which occurred in the II chromosome and eight of which occurred in the III chromosome. Of the six double crossovers, four were the result of recombinations occurring in regions *J–lt* and *stw–Bkd* of the II chromosome. The other two doubles occurred in adjacent regions of chromosome III, *st–sr–Pr* and *th–st–sr*. The one triple crossover was the result of crossing over in regions *J–lt* and *stw–Bkd–px* of chromosome II.

4. A few crossovers were recovered from the 6–9 day brood, but the majority, 13, were found in the 9–12 day brood. From this distribution it appeared that most crossovers in irradiated males must have been formed in the early stages, probably in spermatogonial cells.

5. A total of 20 translocations were recovered, and of these, 17 were between the II and III chromosomes. The other three showed a chromosomal exchange between the Y and III chromosomes.

6. The major portion of the translocations was recovered in the 3–9 day breeding period. This indicated that translocations were induced in cells which probably were undergoing meiosis and spermiogenesis at the time of irradiation.

7. The frequency of crossovers showed a tendency to be greater at higher doses, maximum frequency 0.20 ± 0.10 percent occurring after 4500r.

8. The translocation frequency also appeared to be larger at higher doses, maximum frequency 3.7 ± 1.63 percent occurring after 4500r.

9. Crossing over occurred primarily in the spindle attachment regions of the two long autosomes.

10. The question of whether one or two ionizations were necessary for induced crossing over to occur was discussed.

11. Crossovers and translocations overlapped in some broods and in some few families, but not to a significant extent.

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A YELLOW PIGMENT IN THE TESTIS OF EPHESTIA: ITS DEVELOPMENT AND ITS CONTROL BY GENES[1]

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IT is generally accepted that genes control the formation of specific substances. It has furthermore been shown that even though a gene may primarily control one specific substance, a large number of chemical processes may be secondarily affected, so that a complicated pattern of pleitropic gene effects is observed. One of the goals of physiological genetics is to arrange the different effects of a single gene substitution into an order of dependent causes. This applies to biochemical as well as morphological pleiotropic gene effects.

Most of the material on which biochemical genetics is founded is derived from work with microorganisms. In higher organisms, the control of specific substances by genes has also been observed. In many cases, a complex pleiotropic pattern of gene effects on the chemical constitution of the organism has been found. The gene *a* in Ephestia, e.g., seems to inhibit primarily the oxidation of tryptophan to kynurenin. As a consequence, the pigments derived from kynurenin cannot be formed, and tryptophan is stored in excessive amounts, predominantly in the proteins (CASPARI 1946). Further chemical effects of this gene have been found: on the amount of free amino acids in the hemolymph (CHEN and KÜHN 1956), the quantity of certain fluorescent substances in the head (HADORN and KÜHN 1953), and the presence and quantity of certain fluorescent substances in the abdomen (HADORN and EGELHAAF 1956). In the case of the effect of the gene *a* on the fluorescent substances in the head it has been shown by KÜHN and EGELHAAF (1955) that it is a secondary consequence of the lack of kynurenin caused by the gene. Examples of complex pleiotropic effects on the chemical composition of Drosophila have been investigated by HADORN and his collaborators (HADORN 1956).

In higher organisms, genes may not only control the presence or absence and the quantity of specific substances but also their distribution in space and time. As already mentioned, the effect of the gene *a* in Ephestia on the pattern of fluorescent substances in the head and in the abdomen is different (HADORN and EGELHAAF 1956). Furthermore, it has been found that the fate of certain substances during development may be profoundly influenced by genes. It has been shown in the work of HADORN and MITCHELL (1951) and HADORN (1954) that in Drosophila, isoxanthopterin starts to be formed during the third larval instar, and increases in amount in the pupa. It appears to be particularly concentrated

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in the testes. Many genes are known which influence the amount of isoxanthopterin. In the mutants *w* and *bw*, isoxanthopterin is formed in the pupa, though in reduced amounts, but it disappears shortly after hatching from the pupa, so that it is missing in the adult. It is completely missing in all stages of development in ry^2 (HADORN and SCHWINCK 1956). Cases in which genes determine differences in the time of appearance and disappearance of specific substances appear of particular interest from the point of view of development. It has been earlier proposed (GOLDSCHMIDT 1938) that genes may exert their effects in development by controlling the accurate timing of embryological processes.

In the testis of Ephestia, a yellow pigment appears during the last larval instar and is present through most of the pupal stage. It is absent in the early larval and in the adult testis. The present communication is concerned with attempts to define the nature of this pigment, with the description of the changes in its concentration during development and with strain differences in the developmental fate of the pigment.

MATERIALS AND METHODS

The Ephestia cultures used were kept in finger bowls or in plastic refrigerator jars and fed yellow cornmeal. The animals used in the experiments were kept in a constant temperature room at 25° ± 1° C. Animals were isolated in the instar before last or as old last instar larvae, and checked at daily intervals, or, if need be, at six hour intervals for the occurrence of the last molt or of pupation, respectively. In this way, the age of every animal with respect to a fixed event in development was established.

The strains used were the following:

BII: A wild type strain homozygous for the gene *Rt* which was isolated from material originally obtained from DR. P. W. WHITING, and inbred by brother-sister matings for 93, 94, and 95 generations.

R: Another wild type strain isolated from material obtained from DR. WHITING, homozygous for the gene *rt*, inbred by brother-sister matings for 91, 92, and 93 generations.

aa: The original red-eyed strain homozygous for the gene *a*, inbred by brother-sister matings for 76 generations.

aaO: A strain "isogenic" with BII except for the gene *a* in homozygous condition. Animals of the strain "aa" had been outcrossed to BII, and *aa*-animals were recovered in F_2. These again were outcrossed to BII, so that in the course of time the strain should have the same residual genotype as BII except for a small chromosome piece containing the gene *a*. The animals used in the present study came from the 32nd and 34th generation of outcrossing.

aaC: A red-eyed strain isolated from material obtained through the courtesy of DRS. D. S. GROSCH and R. L. SULLIVAN of the University of North Carolina, Raleigh, N. C. The gene responsible for the eye color is an allele of *a*. The average eye color in this strain is darker than that of aa and aaO, and the testes show more frequently traces of pigment. Whether this difference is due to the presence of a

different allele at the *a*-locus, similar to a^k, or whether it is due to modifiers, has not been established.

wa: A white-eyed strain homozygous for the gene *wa* obtained through the courtesy of PROFESSOR ALFRED KÜHN, Max Planck Institut für Biologie, Tübingen, and inbred in our laboratory by brother-sister mating for 42 and 43 generations.

Paper chromatography was carried out in an ascending direction according to the method of HADORN and MITCHELL (1951) for isolated organs. Testes were dissected out in insect Ringer, cleaned from adhering fat body, transferred with as little fluid as possible to the starting line of a sheet of Whatman No. 1 filter paper and crushed with a glass rod. In some cases, testes were extracted with water or aqueous alcohols, and the resulting solutions were transferred to the starting line. After drying in a dark box, in order to avoid decomposition by light, the paper sheets were rolled into cylinders, as described by HADORN and MITCHELL, and placed upright into a pyrex cylinder which contained at the bottom a dish with the developing solvent. The following solvents were used for development: n-propanol + 1 percent ammonia 2:1, n-propanol + 1N acetic acid 3:1, n-butanol + acetic acid + water 4:1:5, tertiary butyl alcohol + pyridine + water 10:3:7, 3 percent NH_4Cl. On every sheet, one or more drops of an aqueous or alcoholic solution of riboflavin (Riboflavin Merck) were run as controls. All operations were carried out in the dark or under a red light. After development, the paper was dried at room temperature and observed under an ultraviolet lamp.

Riboflavin was determined microbiologically, according to the method of SNELL and STRONG (1939), using *Lactobacillus casei* e 7469 ATCC. The testes of animals of known age were dissected out and the pigment extracted with 5, 10 or 15 ml glass distilled water in the dark. The amount of water used depended on the amount of pigment present in the individual testis, as judged by visual observation. After the pigment was dissolved, 5 ml of the solution was added to 5 ml Bacto Riboflavin Assay Medium "Difco" Certified; in cases where 10 or 15 ml water had been used, the solution was distributed to two or three tubes of medium, respectively, so that in those cases the determinations were carried out in duplicate or triplicate. The riboflavin content of whole pupae was determined by crushing the animals in 10, 15, or 20 ml of water, and adding 5 ml aliquots of the extract to 5 ml riboflavin assay medium. With every experiment, standards containing known amounts of riboflavin (Riboflavin Merck) were run. After sterilization, the tubes were inoculated in the dark room under a red light in order to prevent photolysis of riboflavin and incubated for 72 hours at 37°C. The amount of growth was determined by titration with .1N NaOH.

RESULTS

Nature of the pigment: The pigment is insoluble in ether and chloroform, and dissolves very slowly in absolute alcohol. It dissolves more rapidly in water, and in alcohol or acetone containing water. It disappears quickly in the light, particularly fast in alkaline solution, but is stable in the dark in neutral or acid solution.

The results of paper chromatography of aa testes in different solvents are represented in Table 1. Since some variability of Rf values exists, each value is the mean of at least 19 chromatographs. The number of fluorescent substances found is relatively small. Two well-defined spots appear in most solvents and a third one sometimes, but not always in the first three. With 3 percent NH_4Cl, no clear separation of spots is obtained, but a fluorescent smear is found, with yellow fluorescence in the more advanced part and a more greenish fluorescence in the

TABLE 1

Mean Rf values and fluorescence colors of fluorescent substances, from testes of aa pupae and prepupae in different solvents

Spot	n-propanol + 1% NH_4OH 2:1		n-propanol + 1N acetic acid 3:1		n-butanol + acetic acid + water 4:1:5		tertiary butanol + pyridine + water 10:3:7	
	Rf	color of fluorescence	Rf	color of fluorescence	Rf	color of fluorescence	Rf	color of fluorescence
3	.58	blue-green	.68	green-blue	.64	green-blue		
2	.41	yellow	.38	yellow	.30	yellow	.61	yellow
1	.19	blue-violet	.28	light blue	.20	blue-green	.46	blue-violet
Riboflavin	.40	yellow	.39	yellow	.30	yellow	.61	yellow

least advanced part. Spot two had in all cases an intense yellow fluorescence, and is frequently yellow in visible light. Its Rf value is in all solvents identical with that of the control riboflavin. If the extracted aqueous pigment is exposed to light and chromatographed with n-butanol-acetic acid-water (4:1:5), the yellow spot with Rf .30 disappears, and a faint greenish spot with an Rf .64 is found. The same result is obtained when riboflavin is exposed to light. If propanol-1 percent ammonia is used as a solvent, the corresponding Rf values are .40 for the yellow spot and .56 for the green-blue spot appearing after the pigment or riboflavin has been exposed to light. The green-blue spots three with high Rf values indicated in Table 1 are found only in occasional experiments. They may represent a breakdown product of the yellow fluorescent substances. Spot one varies in the color of its fluorescence from blue-green to blue and blue-violet, depending on the solvent. It may, therefore, not be an identical substance in all instances. In addition, a number of indistinct faint blue or green spots of low Rf values (.03–.09) are occasionally found in individual cases.

At least one component of the visibly yellow pigment seems to be contained in the yellow fluorescent spot. Its solubility, its sensitivity to light, and its behavior in paper chromatography with all solvents used for development agree with the hypothesis that the pigment might be riboflavin.

In view of the fact that many pteridines show a similar solubility and sensitivity to light, and cannot be distinguished from riboflavin by paper chromatography in at least some of the solvents (FORREST and MITCHELL 1954a, b; VISCONTINI *et al.* 1955a), an attempt was made to use the extracted pigment as a substitute for riboflavin in the nutrition of *Lactobacillus casei*. It was found that *Lactobacillus*

casei can grow on pigmented extracts of the testis as a source of riboflavin. It may therefore be suggested tentatively that the pigment is at least in part riboflavin.

It might be objected that pteridines might be able to substitute for riboflavin in microbiological tests, particularly since a pteridine structure forms part of the riboflavin molecule. This possibility seems to be contradicted by the observation of DANIEL, NORRIS, SCOTT and HEUSER (1947) that eight different pteridines inhibit the growth of *Lactobacillus casei* in complete medium, apparently by competitive interference with the activity of folic acid. All substances used by DANIEL *et al.* are 2-4-diaminopteridines, while those isolated from insects, particularly from Drosophila, by FORREST and MITCHELL (1954b, 1955) and VISCONTINI *et al.* (1955a, b) and from Bombyx by NAWA and TAIRA (1954) are 2-amino-4-hydroxy-pteridines. It was therefore attempted to determine whether one of the 2-amino-4-hydroxy-pteridines, xanthopterin (Delta Chemical Works) can be used as a substitute for riboflavin in the growth of *Lactobacillus casei*. The results were negative. No growth occurred in cultures containing xanthopterin instead of riboflavin. If xanthopterin was added to medium enriched with riboflavin, inhibition was present in high concentrations of xanthopterin. In low concentrations of xanthopterin (.2 μg riboflavin + .2 μg xanthopterin) the inhibition found—if any—was slight, below five percent, and insignificant. It appears likely therefore that pteridines cannot substitute for riboflavin. All evidence available points to the conclusion that riboflavin is contained in the yellow pigment. Final confirmation will be possible only when purified samples of the pigment have been obtained.

Chromatograms from testes of wild type pupae and prepupae of the strains BII and R show the same fluorescent spots that are regularly found in aa pupae and prepupae. The wa-strain, on the other hand, does not show any fluorescent substances, except in some cases a very indistinct purple streak. This result is in agreement with the findings of HADORN and KÜHN (1953) and KÜHN and BERG (1956) that in wa Ephestia heads the number and amount of fluorescent substances are strongly reduced.

The testis sheath of Ephestia, like that of all Lepidoptera, consists of two histologically different layers of cells. The outer one consists of several layers of large, irregularly arranged cells and is called "membrana communis" by OMURA (1936) and "tunica externa" by DA CUNHA (1942). The inner layer, the "capsula lobuli" of OMURA and "tunica media" of DA CUNHA, is a connective tissue layer continuous with the septa dividing the follicles; the ommochrome pigment of the testis sheath is restricted to this layer. The yellow pigment is found in the tunica externa cells in the form of short, thick, distinctly yellow rods (Figure 1). The tunica externa which is sharply delimited from the body cavity in the young larva, becomes thicker and less distinct in its boundary during the last larval instar and the prepupa. The individual cells become larger, their cytoplasm stains less distinctly, and they contain osmiophilic droplets, three of which can be seen, though out of focus, in Figure 1. During the same period, the yellow rodlets appear. This state of the external layer persists through most of the pupal testis, but

in the later pupa the layer becomes much thinner and flattens out, becoming again sharply delimited from the hemolymph and losing its yellow color.

The fact that the riboflavin is found in the form of particles recalls the finding of TSUJITA (1948), that riboflavin in Bombyx is found in the Malpighian tubule cells in the "chromoplasts." The crystal-like shape of the chromoplasts in Bombyx, as shown in a photomicrograph given by KIKKAWA (1953), is, however, different from the plump rodlets found in the testis of Ephestia. TSUJITA (1948) claims that the chromoplasts develop from chondriosomes, i.e., mitochondria.

Development of the pigment: The results of the microbiological determinations of riboflavin in isolated testes of Ephestia are summarized in Table 2 and in Figures 2 and 3. The general picture is similar in all strains with the exception of wa. The amount of riboflavin in the testis increases during the last larval instar, first gradually, later steeply. In the pupa it reaches a plateau of more or less long duration, and decreases again in the older pupa. In details, however, significant differences are found between the different strains.

In the R-strain, riboflavin in the testis increases slowly during the last larval instar, steeply in the prepupa. It keeps a plateau in the early pupa, up to six days and decreases gradually to the adult stage. BII has a similar pattern of develop-

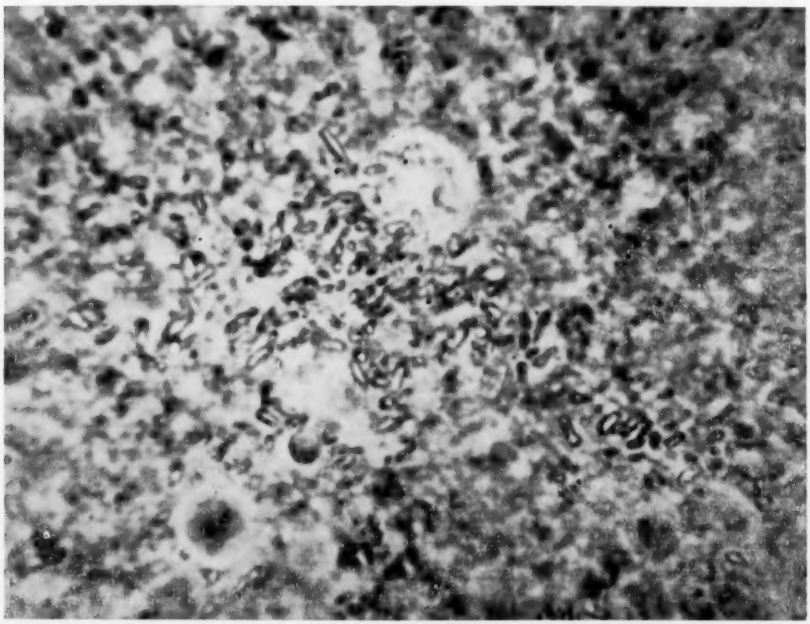

FIGURE 1.—Testis sheath of aaO pupa spread out in saline. Phase, 1600×. Yellow rodlets. Three fat droplets in center and lower left.

TABLE 2

Riboflavin content of individual testes from different strains at different stages of development (μg riboflavin)

Stage \ Strain	R Mean±SE	n	BH Mean±SE	n	aeO Mean±SE	n	aaC Mean±SE	n	aa Mean±SE	n	wa Mean±SE	n
Instar before last	.....		.091±.0002	6	.091±.0003	7	.....		.....			
After last molt							.....		.....			
0–2 days	.....		.002±.0002	11	.002±.0003	10	.....		.....			
2–4 days	.003	2	.004	1	.007±.002	8	.007	2	.....			
4–6 days	.007±.001	17	.011±.002	8	.016±.001	29	.019±.002	25	.....		.004±.0003	18
6–8 days	.025±.005	15	.076±.015	12	.057±.005	25	.059±.011	12	.....			
prepupa	.120±.017	8	.102±.012	5	.149±.017	6	.186±.013	7	.181±.030	8		
After pupation												
0–2 days	.158±.007	21	.193±.004	71	.268±.010	24	.254±.010	19	.312±.010	32		
2–4 days	.142±.008	6	.180±.005	24	.272±.007	21	.264±.007	42	.308±.005	50		
4–6 days	.159±.007	19	.204±.005	29	.228±.008	19	.231±.011	24	.260±.017	14		
6–8 days	.136±.004	61	.234±.005	30	.257±.004	45	.168±.017	23	.142±.011	26	.006±.0004	21
8–10 days	.111±.004	46	.176±.007	46	.222±.011	28	.054±.009	21	.055±.005	32		
10–12 days	.073±.005	33	.115±.005	114	.080±.004	69	.009±.0004	51	.009±.001	5		
over 12 days	.035	3	.034±.003	39	.025±.004	13	.007±.0012	7	.008	4		
Adult	.023±.004	8	.014±.002	20	.008±.003	18	.006±.0004	19	.....		.004±.0003	10

ment, but with some striking differences. BII has a higher amount of riboflavin than R, starting at 6–8 days after the last larval molt ($t = 3.145$, $df = 27$, $P < .01$) and up to 12 days after pupation ($t = 5.940$, $P < .001$). A second characteristic of BII is the appearance of a peak in riboflavin content in the pupal period at 6–8 days after pupation. The difference between the values at 4–6 days and at 6–8 days is significant, $.030 \pm .0078$ μg ($t = 3.841$, $df = 58$, $P < .01$).

The difference between aaO and BII must be ascribed to the gene *a* or a chromosome piece very closely linked to it, since the strains are "isogenic" except for the gene *a*. Its effect may be described in the main as a significantly higher level at the plateau throughout most of the pupal period. The peak at 6–8 days is present in aaO as it is in BII and significant ($t = 3.353$, $df = 63$, $P < .01$). In addition, there is a significant drop in riboflavin value at 4–6 days ($t = 4.139$, $df = 39$, $P < .001$). No indication for a drop at the same time is present in BII.

aaC differs from aaO particularly by the fact that the second peak at 6–8 days after pupation is missing. Since this peak does not appear either in aa whose *a*-allele is the same as that of aaO, it must be assumed that the peak at 6–8 days is due to the genetic background of BII. Both aaC and aa are similar in the high riboflavin values attained and in the general shape of the curve, resulting in a

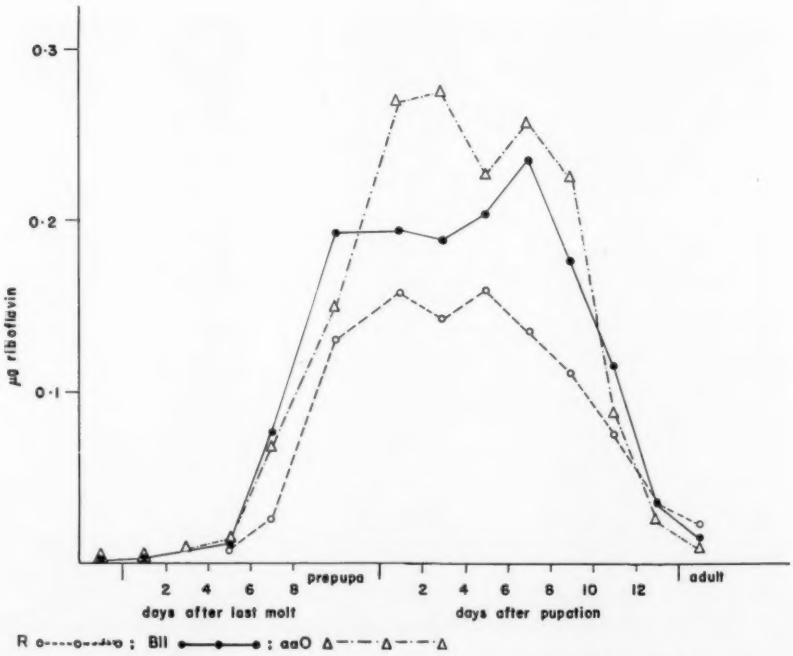

FIGURE 2.—Riboflavin content of Ephestia testes during the last larval instar and in the pupa.

significant drop as early as 4–6 days after pupation ($t = 2.538$, $df = 65$, $P < .02$ for aaC and $t = 2.709$, $df = 63$, $P < .01$ for aa). The only difference between the two strains is a significantly higher level of riboflavin in aa as compared with aaC testes in the early pupa ($t = 4.101$, $df = 50$, $P < .001$ for 0–2 days, and $t = 5.115$, $df = 91$, $P < .001$ for 2–4 days).

Gene *a* seems therefore to determine a higher riboflavin level in the pupal testis than its allele a^+. This is borne out by the consistently higher values found in the three *aa* strains as compared to the two a^+a^+ strains, as well as by the difference between the isogenic strains aaO and BII. It is also suggested that the drop in riboflavin level occurs earlier in strains homozygous for *a* than in those homozygous for its wild type allele. Here again, the similarity of all three aa strains and the difference between aaO and BII form the basis of the suggestion. In aaO, this drop is statistically significant at 4–6 days, but is somewhat obscured by the second peak occurring at 6–8 days which is found only in BII and aaO and is therefore ascribed to the genetic background of BII.

The wa strain is characterized by exceedingly low values of riboflavin, comparable to the levels found in the beginning of the last larval instar in the other

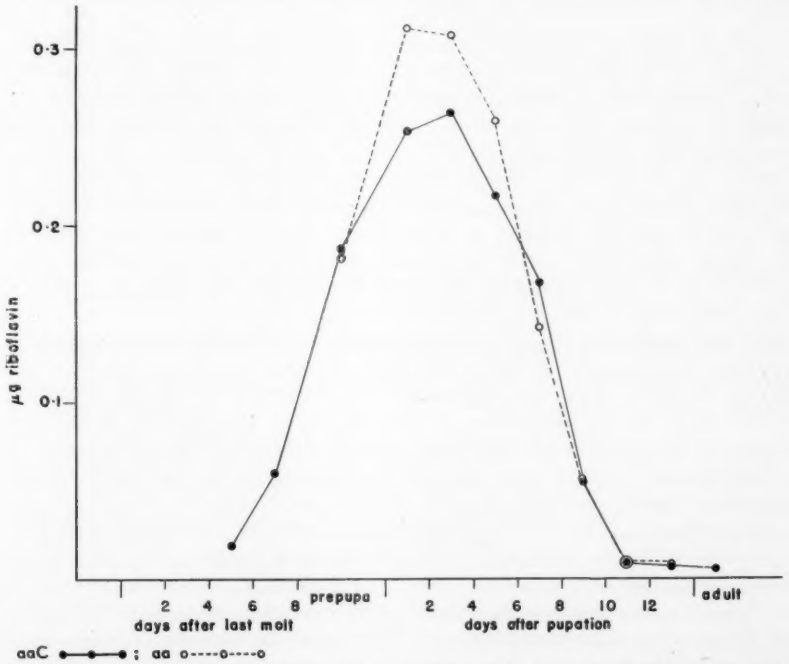

FIGURE 3.—Riboflavin content of Ephestia testes in the late larva and prepupa, and in the pupa.

strains. Some indication for an increase in the pupal stage is present even in wa. but this may not be considered as certain, since the values obtained with low levels of riboflavin are subjected to a considerable error of measurement.

Since in wa the amount of riboflavin in the testis is strongly reduced, the question arises whether this is due to a general reduction of riboflavin in wa animals, or whether it is due to an inability to concentrate the riboflavin in the testis sheath. For this reason, wa and aaO pupae, 7–10 days old, of both sexes, were compared with respect to their content of riboflavin. The results are shown in Table 3. Even though the number of animals used is low, the difference between

TABLE 3

Riboflavin content of aaO and wa total pupae

Strain	Age (after pupation)	Sex	Mean±SE (μg riboflavin)	n
aaO	7–10 days	♂ and ♀	.526±.023	6
wa	7–10 days	♂ and ♀	.174±.014	4
aaO	2–4 days	♂ without testis	.240±0.12	12
wa	2–4 days	♂	.252±.007	12

the two strains is striking: aaO pupae have about three times as much riboflavin as wa pupae. No differences between the sexes appear in either strain. In order to find out how much of this surplus in aaO pupae is due to the material contained in the testes, wa males were compared with aaO males whose testes had been removed. For this experiment, younger pupae (2–4 days) were chosen, since in the older pupae part of the riboflavin becomes concentrated in the Malpighian tubules. No difference appears between the two strains at this stage if the testes of aaO are removed; in other words, the total difference between the two strains at 2–4 days after pupation is due to the riboflavin content of the testis which, if the values of Tables 2 and 3 are combined, amounts to over 50 percent of the total riboflavin present in the pupa.

The disappearance of the pigment from the testis: The fact that the riboflavin contained in the testis disappears in a relatively short time in the middle to late pupa, raises the question, what becomes of the riboflavin removed from the testis? Visual observation suggested that at the time when the pigment in the testis disappears, the Malpighian tubules assume a yellow color. Microscopically, the yellow pigment in the Malpighian tubules appears in the form of slender rodlets or needles, thinner than the pigmented rods in the testis sheath, and more similar to the needles found in the Malpighian tubules of Bombyx (TSUJITA 1948; KIKKAWA 1953). The appearance of yellow intracellular needles in the Malpighian tubules of the late pupa of Ephestia has been observed by WOLFRAM (1948).

In pupae of strain BII, 95th generation of inbreeding, testes and Malpighian tubules were removed during the second part of the pupal period and their riboflavin contents determined. The results are represented in Figure 4. Each point in Figure 4 is the mean of nine or more determinations. The absolute values of

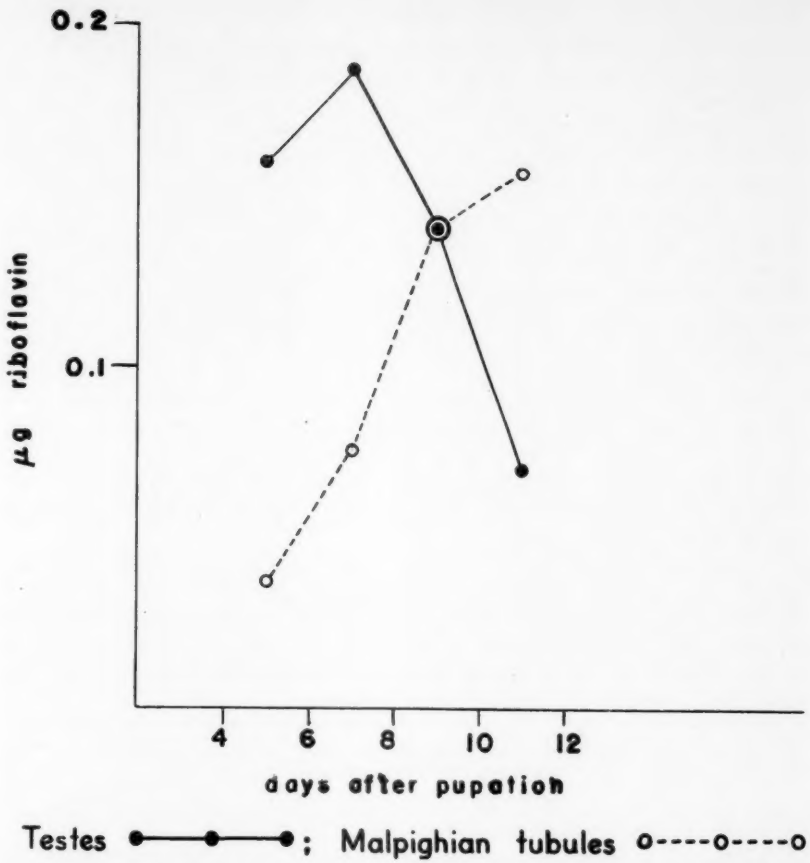

FIGURE 4.—Riboflavin content of testes and Malpighian tubules during later pupal development of BII Ephestia.

riboflavin found in the testes are lower than those found in the earlier experiment (Figure 2), but the general shape of the curve is similar, including the sharp peak at 6–8 days. The curve for riboflavin in the Malpighian tubules is the reverse of that in the testes: as the pigment in the testes disappears, it increases in the Malpighian tubules. Only the rise in the testes at 6–8 hours does not seem to be reflected in the Malpighian tubules. The correlation between the amount of riboflavin in the testes and in the Malpighian tubules of individual pupae is indicated by a correlation coefficient of $r = -.511$ for 45 df, a highly significant value. The general result suggests the interpretation that pigment removed from the testis may be deposited in the Malpighian tubules.

DISCUSSION

It has been assumed, in the preceding pages, that the yellow pigment in the tunica externa of the testis sheath is at least in part riboflavin. This conclusion is based not only on the fact that it resembles riboflavin in its chemical characteristics, including the Rf values with all developing solvents tested, but in particular on the fact that it can serve as a source of riboflavin for *Lactobacillus casei*. In the microbiological riboflavin determinations, the fact should be taken into consideration that, according to the chromatograms, the extracts of the testes contain other fluorescent substances which may be pteridines. Since pteridines inhibit the growth of *Lactobacillus casei*, the values for riboflavin obtained may be low. The results with xanthopterin indicate, however, that the inhibition should not be so high as to obscure the results, since it may be assumed that the pterins are present in relatively low concentrations, and the inhibitory effect of xanthopterin becomes significant only when high concentrations are used (20 percent for 30 μg xanthopterin). Furthermore, in the experiments involving whole pupae, water had been used for the extraction of the riboflavin. Since part of the riboflavin may be present in conjugated form, the extraction may not have been complete, and the riboflavin values obtained would be too low. The difference in riboflavin content of wild type and wa pupae established by this method is, however, so large that it may be regarded as real.

The presence of riboflavin in the testis of adult Ephestia has been demonstrated by EGELHAAF (1956). The pattern of fluorescent substances found by EGELHAAF in chromatograms of adult Ephestia testes is richer than that described in the present paper. This may in part be due to the fact that he used extracts of the organ, while in the present work crushed organs were chromatographed. This procedure would favor the extraction of substances contained in the outermost layer of the testis, such as the yellow pigment investigated. A comparison with EGELHAAF's Rf-values suggests, however, that with propanol-ammonia as a solvent, our spot two may correspond to EGELHAAF's spot Ri (riboflavin), our spot one to EGELHAAF's spots (f)h and g (xanthopterin and isoxanthopterin). Our spot three may be identical with EGELHAAF's spot p which, just as in our material, was found only in some of the chromatographs. Some other spots were only rarely and indistinctly present in our material. In the Malpighian tubules of adults, EGELHAAF (1956) could demonstrate the presence of riboflavin in only one instance. In view of the considerable amounts of riboflavin found in the Malpighian tubules of the late pupa, it may be suggested that this substance may be excreted soon after hatching. The presence of riboflavin is well established for the Malphighian tubules of Bombyx (TSUJITA 1948) and other Lepidoptera (DRILHON and BUSNEL 1939). In Bombyx, as in Ephestia, it is present in the form of cytoplasmic structures. A difference between the riboflavin content in the Malpighian tubules of Bombyx and Ephestia is that in Bombyx it is already clearly visible in the last larval instar, whereas in Ephestia it becomes apparent only in the second part of the pupal period. It may be added that both wild type strains investigated, BII and R, are devoid of ommochrome pigment in the Malpighian

tubules and therefore probably homozygous for the gene *om*. This confirms WOLFRAM's (1948) conclusion that the genes controlling the presence of ommochrome in the Malpighian tubules are not identical with *Rt* and *rt*.

The question whether there is any connection between the appearance of riboflavin in the tunica externa and the formation of ommochrome pigment in the tunica media of the testis may be raised. Such a possibility is suggested by the fact that the time during which riboflavin is found in the testis corresponds approximately to the period during which active synthesis of the ommochrome pigment takes place (CASPARI 1955). Furthermore, both mutants affecting the amount of the ommochrome pigment in the testis affect also its riboflavin content, though in opposite directions. KIKKAWA (1943) has similarly suggested a relation between ommochrome pigments and riboflavin in Bombyx.

The possibility that riboflavin may be involved in one of the steps leading to ommochrome formation, is suggested by experiments by CHARCONNET-HARDING, DALGIESH and NEUBERGER (1953) and by MASON (1953) with the rat. If riboflavin deficient rats are fed tryptophan, urinary excretion of anthranilic acid, kynurenic acid, xanthurenic acid and occasionally of kynurenin and 3-hydroxykynurenin is observed. CHARCONNET-HARDING *et al.* suggest that riboflavin deficiency may affect the steps leading from kynurenin to 3-hydroxykynurenin. These steps occur in the chain of reactions leading to ommochrome pigment. Ommochrome synthesis may, however, proceed without the presence of conspicuous accummulations of riboflavin, as in the eyes of Ephestia, where the maximum amount does not exceed .0025 μg per head (REISENER-GLASEWALD 1956). According to REISENER-GLASEWALD, the amount of riboflavin in the Ephestia eye decreases gradually during pupal development and disappears completely in the late pupa.

The finding of a difference in the riboflavin content of the testis between a^+ and *a* strains increases the already considerable number of substances known to be influenced by the gene *a* (CASPARI 1946; HADORN and KÜHN 1953; CHEN and KÜHN 1956).

Gene *wa* is characterized by the suppression of many fluorescent substances (HADORN and KÜHN 1953; KÜHN and BERG 1956); the reduction in the total amount of riboflavin and its almost complete absence in the testis are in line with this observation. The question arises whether this lack of riboflavin is due to an inhibition of its formation, to an interference with its absorption or its destruction in wa animals. Nutrition experiments by FRAENKEL and BLEWETT (1946) have shown that larvae of *Ephestia kühniella*, in contrast to those of *E. elutella*, can develop in the absence of riboflavin, suggesting that they are able to synthesize this vitamin. All three explanations must therefore be considered possible.

The relation of the lack of riboflavin to the inhibition of ommochrome formation in wa is unclear. HANSER (1948) has proposed that the primary effect of the gene *wa* is an inhibition of the formation of precursor granules on which the pigment is deposited. The basis of this hypothesis is the observation that precursor granules are lacking in *wa wa* eyes. But since it has been found that no precursor

granules are present in colorless *aa* testes, and that in *aa* testes the appearance of precursor granules is closely connected with the first appearance of ommochrome pigment, (CASPARI and BLOMSTRAND 1956), the absence of precursor granules in *wa* may also be a consequence of the lack of pigment. The possibility that gene *wa* affects pigment primarily by means of its effect on riboflavin cannot be excluded on this basis. REISENER-GLASEWALD (1956) found, however, that in the pupal eyes of *wa* animals the amount of riboflavin is higher than in the pigment forming strains. Just as in the latter strains, it decreases during pupal development and finally disappears, but shows a significant peak in the late pupa, shortly before its disappearance.

Effects of genes affecting the eye pigmentation on the amount of riboflavin are also known from Drosophila. In Drosophila, riboflavin is contained in HADORN and MITCHELL's (1951) spots Fl 4/5, together with three other substances all of which appear to be pteridines (HADORN and SCHWINCK 1956). This spot is missing in the mutants *bw* and *w*, indicating that riboflavin as well as the other substances are lacking in animals homozygous for these genes. There are many other eye color mutants in which the intensity of Fl 4/5 is increased (*cl*, *se*, *sed*, ry^2, and also *l*, *ca*, pn^2) or reduced (w^a, w^e, g^2, p^p, *lt*, *car*, *pr*), as compared to the wild type (HADORN and MITCHELL 1951; HADORN and SCHWINCK 1956). In some of these cases (e.g. *se*, FORREST and MITCHELL 1954a) it is known that a yellow pteridine is accumulated; but it is uncertain whether the amount of riboflavin is also affected by these genes.

Genetic strain differences affect not only the quantity of riboflavin present in the testis, but also the timing of its disappearance. The pattern of its appearance during the last larval instar is very similar in all five strains investigated, even though the time of formation of ommochrome is different for BII and R (CASPARI 1955). Gene *a* appears to be responsible for an earlier disappearance of riboflavin from the testis than in a^+ animals. How this character can be integrated with the other phenes associated with *a* is not clear. Furthermore, there is a genetic condition in BII which causes riboflavin in the testis to increase at 6–8 days after pupation. Both observations suggest that these genetic conditions affect biochemical processes in particular types of cells at a sharply defined time of development.

SUMMARY

1. A yellow pigment appearing in the testis of Ephestia is described. The pigment is deposited in rodlets in the cells of the outer layer of the testis sheath.

2. It has been tentatively concluded that the pigment is at least in part riboflavin on the basis of its solubility, its Rf values in paper chromatography (using a variety of solvents), and its ability to supply the riboflavin requirement of *Lactobacillus casei*.

3. The pigment appears gradually in last larval instar larvae. Its amount in the testis rises steeply in the prepupa, reaches a plateau in the early pupa and disappears in the late pupa. At the time of its disappearance riboflavin becomes deposited in the Malpighian tubules.

4. Strain differences in the amount of the pigment and the pattern of its disappearance have been found. Some of them can be ascribed to known genes.

5. Gene *a* causes the appearance of larger amounts of riboflavin in the testis sheath than a^+. There is also some indication that it causes an earlier disappearance of the pigment from the testis.

6. Strain BII and the aa strain, isogenic with it, are characterized by a peak in the amount of riboflavin in the testis 6–8 days after pupation. This peak is ascribed to the genotypic background of BII.

7. In wa testes, no yellow pigment is visible, and the amount of riboflavin is strongly reduced. The total amount of riboflavin in young wa pupae is reduced as compared to young aa pupae by the amount present in aa pupal testes.

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TRANSPOSITION AND THE STABILITY OF MODULATOR IN MAIZE[1,2]

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THE variegated pericarp allele (P^{vv}) in maize has been pictured as a dual structure comprising P^{rr}, a gene for self-red pericarp and cob, and Modulator *(Mp,)* a separable element which when present at the *P* locus in conjunction with P^{rr}, suppresses the pigment-producing action of the latter. P^{vv} mutates frequently in somatic tissue to stable self-color, giving the disorderly pattern of red sectors on a colorless background characteristic of the variegated pericarp ear. The mutation of P^{vv} to P^{rr} involves transposition of Modulator from the *P* locus to one or another, usually unknown, position elsewhere in the genome. An effect of such a transposed Modulator *(tr-Mp)* in a variegated plant is to reduce the number of P^{vv} to P^{rr} mutations by somewhat more than one half, thus changing the collective phenotype of the ear from "medium" to "light" variegated (BRINK and NILAN 1952; BRINK 1954).

The strong modifying action of transposed Modulator on P^{vv} to P^{rr} mutations was used as a criterion in the present study to determine whether Modulator elements derived by independent transpositions from a common P^{vv} allele, on a given genetic background, were alike.

MATERIALS AND METHOD

It was desired to produce, on a uniform genetic background, plants carrying a single P^{vv} allele from a common source and hemizygous for a series of transposed Modulators derived by independent mutations of P^{vv} (separate transpositions of *Mp* from the *P* locus to other chromosome sites) within a single stock. These plants would be of the composition P^{vv}/P^{vv} (or P^{vr}) + *tr-Mp*/—, and hence would be light variegated. The frequency of mutation to P^{rr} of the P^{vv} allele common to these light variegateds would be expected to reflect in each instance the modifying action on the P^{vv} to P^{rr} event of the particular transposed Modulator present.

Plants of the desired composition were produced in two steps from inbred foundation lines already at hand. First, colorless pericarp individuals hemizygous for transposed Modulator were identified in families in which they would be expected to occur rather frequently by means of the *Ds* test (MCCLINTOCK 1951; BARCLAY and BRINK 1954). Secondly, all these plants were pollinated with a single com-

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posite collection of pollen from a highly inbred P^{vv}/P^{vv} stock. The light variegated offspring resulting from the latter matings provided the ears desired for scoring.

A P^{vv} allele previously had been incorporated into two inbred lines of dent corn, 4Co63 and W23. Following the initial outcrosses the P^{vv}/P^{ww} and P^{vv}/P^{wr} heterozygotes had been backcrossed six times to the respective inbreds. Newly arisen, independently occurring light, and very light variegated mutants were selected at harvest in the fifth backcross generation to serve as parents for the next generation. These two phenotypes have the following constitution: light variegated, P^{vv}/P^{ww} (or P^{wr}) + *tr-Mp* and very light variegated, P^{vv}/P^{ww} (or P^{wr}) + *tr-Mp* + *tr-Mp*. They were expected to give by segregation in the next backcross generation the genotypes desired for the present experiment viz., P^{ww}/P^{ww} + *tr-Mp* and P^{wr}/P^{wr} + *tr-Mp*. Both the 4Co63 and W23 inbred lines carry the recessive gene *c*, for colorless aleurone.

Material in 4Co63 genetic background: Progenies containing plants of the desired class were obtained from five light variegated and three very light variegated ears. Fifteen plants were tagged at random in each family, and pollen from each of these plants was applied to the silks of (1) 4Co63 plants and (2) plants of the genotype *A C R / A C R* and heterozygous for *Ds* in the standard position on chromosome 9 (McCLINTOCK 1951). The P^{ww}/P^{ww} segregates, among the 15 tagged individuals, which carried a *tr-Mp* were identifiable at harvest since the corresponding test ears on *Ds* stock (resulting from cross 2 above) showed *C* to *c* mottling in the aleurone layer of the endosperm. All the groups of plants, comprising 15 each, were pollinated with a single composite collection of pollen from ten W23 homozygous variegated pericarp plants. Samples of the same collection of pollen were also applied to the silks of four plants of the standard 4Co63 inbred line in order to measure separately the mutations contributed to the testcross offspring by the unstable $P^{vv}P^{vv}$ male parent.

The seeds of the above crosses (P^{ww}/P^{ww} + *tr-Mp*) × (W23 P^{vv}/P^{vv}) and (4Co63 P^{ww}/P^{ww}) × (W23 P^{vv}/P^{vv}) were grown out, and the resulting plants allowed to open pollinate. The light variegated ears (P^{vv}/P^{ww} + *tr-Mp*) were used for scoring the frequency of somatic mutation of P^{vv} to P^{vr}, since these alone carried transposed Modulators derived from the P^{vv} allele in the original 4Co63 P^{vv}/P^{ww} stock.

Material in W23 genetic background: Progenies containing plants of the desired genotype were obtained from five light variegated ears in the W23 P^{vv}/P^{wr} strain. Fifteen plants per family were tagged at random, and pollen from each of these plants was applied the silks of (1) W23 plants and (2) plants of the genotype *A C R / A C R* and heterozygous for *Ds*. The resulting test ears from cross 2, above, were used to identify the P^{wr}/P^{wr} segregates that carried a *tr-Mp*. The 15 tagged plants in each family were pollinated with a single composite collection of pollen from ten W22 homozygous variegated pericarp plants. A sample of the same collection of P^{vv}/P^{vv} pollen also was applied to the silk of four plants of the W23 inbred line, as controls on the male parentage.

The seeds from the above two crosses, ($P^{vcr}/P^{vcr} + tr\text{-}Mp$) × (W22 P^{vv}/P^{vv}) and (W23 P^{vcr}/P^{vcr}) + (W22 P^{vv}/P^{vv}), were grown out, and the resulting plants allowed to open pollinate. At harvest the light variegated ears were chosen for scoring the frequency of somatic mutation of P^{vv} to P^{rr}.

Scoring somatic mutations of P^{vv} *to* P^{rr}: In scoring the frequency of somatic mutation, size of the mutant area was estimated with the aid of models (Wood and Brink 1956). The mutations were grouped into five size-classes, ⅛ to less than ¼; ¼ to less than ½; ½ to less than 1; 1; and 2 or more kernels. These classes were designated by their minimal values.

EXPERIMENTAL RESULTS

Families as large as the seed supply permitted were grown from the two pairs of crosses, ($P^{vcw}/P^{vcw} + tr\text{-}Mp$) × (W23 P^{vv}/P^{vv}) plus control, and ($P^{vcr}/P^{vcr} + tr\text{-}Mp$) × (W22 P^{vv}/P^{vv}) plus the corresponding control. The data obtained on scoring these progenies for pericarp phenotype are presented in Tables 1, 2, and 3.

Table 1

The distribution in the different pericarp phenotypes of the offspring from the cross (P^{ww}/P^{ww} + tr-Mp) × (*W23* P^{vv}/P^{vv})

Transposition number	Family number	Very lt. variegated	Light variegated	Medium variegated	Red	Colorless	Total
1	K3*	15	33	16	10	..	74
	K4	5	28	28	12	..	73
	K5*	26	30	12	9	..	77
	K6	3	28	31	8	1	71
	K7	3	25	22	6	1	57
2	K9	6	37	21	6	1	71
	K10	8	32	25	10	..	75
	K11	4	23	18	8	..	53
3	K12*	30	31	11	6	..	78
	K13*	24	5	22	6	..	57
	K14	4	29	31	11	2	77
	K15	5	27	21	5	..	58
4	K16*	23	8	8	4	..	43
5	K18	4	34	27	2	2	69
	K19	2	34	17	6	..	59
	K20	2	36	22	11	1	72
6	K22	8	40	27	6	1	82
	K23	4	31	19	11	1	66
	K24	1	22	26	8	1	58
	K25	9	37	19	6	1	72
7	K27	3	35	26	11	2	77
	K28	1	32	29	7	1	70
8	K30	4	20	26	5	1	56
	K31	7	35	24	12	1	79

* These families contain a larger proportion of very light variegated ears than normally expected. The probable causes for such excess recovery are discussed in the text.

TABLE 2

The distribution in the different pericarp phenotypes of the offspring from the cross (P^{wr}/P^{wr} + tr-Mp) × (*W22* P^{vv}/P^{vv})

Transposition number	Family number	Very lt. variegated	Light variegated	Medium variegated	Red	Colorless	Total
1	K36*	17	35	14	1	2	69
	K37*	20	32	29	3	1	85
2	K38	3	20	19	..	..	42
	K39	5	30	38	2	..	75
3	K41	4	38	28	2	1	73
4	K43	2	37	33	3	2	77
	K44	7	17	33	..	..	57
	K45	8	27	38	3	..	76
5	K47	8	31	33	..	3	75

* These families contain a larger proportion of very light variegated ears than normally expected. The probable causes for such excess recovery are discussed in the text.

TABLE 3

The distribution in the different pericarp phenotypes of the offspring from the control crosses (P^{ww}/P^{ww} × *W23* P^{vv}/P^{vv}) *and* (P^{wr}/P^{wr} × *W22* P^{vv}/P^{vv})

Cross	Family number	Very lt. variegated	Light variegated	Medium variegated	Red	Colorless	Total
P^{ww}/P^{ww}	K1	..	4	44	5	..	53
×	K2	..	5	26	6	1	38
W23 P^{vv}	K32	..	5	41	8	..	54
	K33	..	8	45	4	..	57
	Total	..	22	156	23	1	202
P^{wr}/P^{wr}	K34	2	6	38	2	..	48
×	K35	..	10	37	..	1	48
W22 P^{vv}	K48	..	7	42	1	..	50
	K49	..	4	35	1	..	40
	Total	2	27	152	4	1	186

Distribution of the three classes of variegated individuals, very light, light, and medium, in the different families are as expected from a light variegated parent, except in five families in Table 1 and two families in Table 2 (marked with an asterisk). There is an excess of the very light variegated phenotype in these families. This phenotype is conditioned by the presence of two transposed Modulators in hemizygous condition, in combination with a P^{vv} allele at the *P* locus (BRINK 1954). It could arise from fertilization of a *tr-Mp* egg by a male gamete carrying P^{vv} plus *tr-Mp*, or result from duplicate transposed Modulators carried either in the egg or the sperm. The occurrence with significant frequency of such duplication of *tr-Mp* is not apparent, however, in the W23 P^{vv}/P^{vv} male parent, as seen by the absence of the very light variegated phenotype in the control families K1, K2, K32, and K33 (Table 3). The presence of very light variegateds

in the control family K34, (Table 3) involving W22 P^{vv}/P^{vv} as male parent, evidences the occasional occurrence of such duplication in these male gametes.

The colorless phenotype, which totals about 1.04 percent among the families in Table 1 and about 1.4 percent among the families in Table 2, is an uncommon class. Its origin may be due to (1) chance contamination or selfing, (2) a mutation of the P^{rr} component of the P^{vv} allele, (3) presence of more than two *tr-Mp* units besides the P^{vv} allele, resulting in complete color suppression, or (4) mutation of P^{vv} to a stable colorless form. The presence of this phenotype in some of the control families (Table 3) in both the 4Co63 and W23 backgrounds suggests that the third possibility is unlikely since it would require that the male gamete carry three *tr-Mp* units at least.

The red-eared plants are the result of mutations of P^{vv} to P^{rr}, interpreted as losses of *Mp* from the *P* locus. The frequencies with which they occur presumably reflect the respective probabilities of microspores having been formed by the parent wherein *Mp* was no longer present at the *P* locus. In the W23 P^{vv}/P^{vv} male parent the proportion is 11.4 percent and in the case of W22 P^{vv}/P^{vv} male parent it is 2.2 percent.

Somatic mutation of P^{vv} *to* P^{rr}

The frequencies of red stripes, interpreted as somatic mutations of P^{vv} to P^{rr} involving loss of *Mp* from the *P* locus, are presented in Table 4 for the eight inde-

TABLE 4

Mutations of P^{vv} *to* P^{rr} *per 1000 kernels on light variegated ears in 4Co63 background, distributed according to the size of the mutant area*

Transposition number	Total number of families examined	Total number of ears examined	Estimated total number of kernels	Number of mutations per 1000 kernels					
				0.25—0.125	0.5—0.25	1—0.5	1	2 or more	Total of all 5 classes
WW segregate + *tr-Mp* × W23 P^{vv}/P^{vv}									
1	5	100	75058	13.91	10.46	6.77	3.97	0.32	35.43
2	3	60	43082	11.23	10.86	6.13	3.25	0.23	31.70
3	4	65	46938	11.36	13.93	9.08	3.20	0.21	37.78
4	1	8	6924	10.98	8.95	7.22	2.89	0.14	30.18
5	3	60	44378	8.90	11.63	6.62	3.31	0.18	30.64
6	4	75	53758	7.81	13.78	6.90	3.74	0.28	32.51
7	2	40	29148	9.19	14.13	8.54	3.05	0.24	35.15
8	2	35	24626	9.01	13.40	9.95	3.70	0.16	36.22

Analysis of χ^2 *test of heterogeneity (frequency of total mutant kernels)*

Class	DF	Calculated χ^2	χ^2 value from table		Significance
			5%	1%	
Transpositions	7	59.019	14.067	18.475	Highly significant
Families within transpositions	16	11.738	26.296	32.000	Not significant

pendent transpositions studied in the 4Co63 background and in Table 6 for the five independent transpositions studied in the W23 background.

It should be pointed out that the light variegated ears, which alone were scored, could have arisen in two ways, (1) fertilization of an egg carrying a *tr-Mp* by a P^{vv} male gamete and (2) fertilization of an egg with no *tr-Mp* by a male gamete carrying P^{vv} + *tr-Mp*. A *tr-Mp* contributed by a male gamete, which is from a different stock, might not be strictly comparable with those present in the eggs. This source of error, arising from mutations of medium to light variegated in the P^{vv} P^{vv} male parent, is small in any case, and can be ignored because it applies uniformly to each group of test families.

The values in the last column of Table 4, based on the frequency of somatic mutation per 1000 kernels, appear to be distributed around two distinct means. The data relating to the eight transpositions in the 4Co63 background have been arranged in Table 5 in accordance with the two groups thus indicated. The "t" test showed that the difference between the values 32.51 (the highest in the lower group) and 35.15 (the lowest in the higher group) was statistically significant, exceeding the critical value, 2.59. The chi-square test for heterogeneity showed that the intergroup difference was highly significant, whereas the intragroup variations were not significant.

TABLE 5

The eight transpositions in the 4Co63 background arranged to form two distinct groups on the basis of total somatic mutation of P^{vv} *to* P^{rr} *per 1000 kernels*

Transposition number	Total number of kernels examined	Total number of mutant kernels	Mutations per 1000 kernels
4	6924	209	30.18
5	44378	1360	30.64
2	43082	1366	31.70
6	53758	1748	32.51
7	29148	1025	35.15
1	75058	2659	35.43
8	24626	892	36.22
3	46938	1775	37.78

Analysis of χ^2 *test for heterogeneity (based on the frequency of mutant kernels) in each of the eight transpositions in the 4Co63 background. The transpositions are divided into two groups*

Source	DF	Calculated value of χ^2	χ^2 value from table		Significance
			5%	1%	
Groups	1	49.84	3.84	6.64	H.S.
Transpositions	6	9.18	12.59	16.81	N.S.
Families within transpositions	16	11.74	26.30	32.00	N.S.

TABLE 6

Mutations of P^{vv} *to* P^{rr} *per 1000 kernels on light variegated ears in W23 background, distributed according to the size of the mutant area*

Transposition number	Total number of families examined	Total number of ears examined	Estimated total number of kernels	Number of mutations per 1000 kernels: 0.25—0.125	0.5—0.25	1—0.5	1	2 or more	Total of all 5 classes
WR segregate + *tr-Mp* × W22 *Pvv/pvv*									
1	2	40	33574	5.30	8.76	3.31	2.03	0.20	19.60
2	2	40	32444	6.10	9.06	4.07	1.23	0.06	20.52
3	1	20	16190	5.25	9.26	3.77	2.10	0.19	20.57
4	3	55	42798	5.72	9.00	3.88	1.61	0.23	20.44
5	1	20	16306	6.13	7.36	3.43	1.66	0.25	18.83

Analysis of χ^2 *test of heterogeneity (frequency of total mutant kernels)*

Class	DF	Calculated χ^2	χ^2 value from table at 5% level	Significance
Transpositions	4	2.504	9.488	Not significant
Families within transpositions	4	3.993	9.488	Not significant

Distribution of the mutations according to size of mutant area

Distribution of the mutations in the five size-classes recorded was examined to see whether the several transpositions differed in this respect. The results of an analysis of variance for the eight transpositions in the 4Co63 background are given in Table 7, and for the five transpositions in the W23 background in Table 8. The differences between the 4Co63 transpositions, referred to in Table 7, are explainable on the basis of variation met with between families within the transpositions, which is highly significant. In Table 8, relating to the W23 stocks, it may be seen that there is no significant difference either between transpositions or between families within the transpositions.

TABLE 7

Analysis of variance (frequency of mutant kernels in different size-classes among the eight transpositions in the 4Co63 background)

Source	DF	SS	MS	Calculated value of F	Value of F from table: 5%	1%	Significance
Transpositions	7	47.44	6.78	7.14	2.01	2.64	H.S.
Families within transpositions	16	43.69	2.73	2.87	1.64	1.99	H.S.
Error	11010	10437.48	0.95	...	...	...	

6.78/2.73=2.48 is not significant for 7 and 16 degrees of freedom.

TABLE 8

Analysis of variance (frequency of mutant kernels in different size-classes among the five transpositions in the W23 background)

Source	DF	SS	MS	Calculated value of F	Value of F from table 5%	Value of F from table 1%	Significance
Transpositions	4	5.53	1.38	1.52	2.37	3.32	N.S.
Families within transpositions	4	3.35	0.84	0.09	2.37	3.32	N.S.
Error	2830	2482.67	0.91	...	...	...	

DISCUSSION

An attempt has been made in the present study to determine whether Modulator when transposed from the *P* locus maintains its stability or undergoes change. A number of Modulator elements derived by independent transpositions from a given P^{vv} allele were compared to see whether they were alike in reducing the frequency of somatic mutation of P^{vv} to P^{rr}. From the data presented in Tables 4 and 5 it is evident that two distinct groups within the eight independent transpositions derived from the 4Co63 stock are recognizable, differing in mutation frequency.

Conceivably the difference in the frequency of somatic mutation exhibited by the two 4Co63 groups of transpositions is due to position effect, and is not the result of change in the Modulator unit itself. BARCLAY and BRINK (1954) and NANCY VAN SCHAIK (1956) previously have shown that Modulator is transposed to various chromosomal sites. Such differences in location possibly may affect the modifying action of *Mp* on the P^{vv} allele. Practical difficulties stand in the way of a definitive test of this hypothesis, but the available evidence makes the explanation unlikely. The rather high frequency of secondary transpositions and the lack of suitable markers in all the chromosome arms pose problems in testing the action of *tr-Mp* at different loci on the frequency of P^{vv} to P^{rr} mutations. It is likely, however, that each of the eight primary transpositions tested in the 4Co63 group involved a different recipient locus initially. Since several ear families were utilized for estimating the effect of each of the transpositions, various secondary transpositions also would have had opportunity to occur. It is highly probable, therefore, that a variety of sites will have been sampled in the present material. Why, then, do the transposed Modulators fall into two groups only, in terms of modifying action on P^{vv} to P^{rr} mutation? An explanation more plausible than position effect is that the two groups in question reflect slightly different forms of Modulator itself. On this basis, seemingly, there is a preference for one other mutant form of the element.

Partition of the eight transpositions in the 4Co63 background into two groups is not referable to a single mutation of Modulator in a generation prior to the isolation of the units for testing. The pedigrees of the eight cases exclude this explanation.

The data in Table 6, pertaining to the transpositions studied in W23 background, show no significant difference between the five cases in frequency of mutation. The integrity of *tr-Mp* apparently was maintained in this background. This observation is in agreement with the earlier observations of WOOD and BRINK (1956) who studied three other independent *Mp* transpositions in the W23 stock in which the frequency of P^{vv} to P^{rr} mutation was found to be alike.

The data relating to distribution of mutations in terms of sector size in the 4Co63 group of transpositions are difficult to account for. It will be recalled that significant differences in this respect between families within each of the two groups were observed, and that no difference between the groups was demonstrable. The only consistent difference between the transposed Modulators in the two 4Co63 groups, therefore, relates to the effect on total frequency of P^{vv} to P^{rr} mutations. The heterogeneity with reference to distribution of mutant size-classes remains unexplained.

The five transpositions studied in W23 background on the other hand, showed similar distributions of the P^{rr} mutants over the different size classes. It may be concluded, therefore, that in this group the five transposed Modulators tested were alike in their effect on distribution during plant development, as well as total frequency, of P^{vv} to P^{rr} mutations.

Modulator differs from conventional genetic units in being transposable from one chromosomal position to another. The present study shows that during such transposition *Mp* maintains its fundamental qualitative character (modification in frequency of P^{vv} to P^{rr} mutations was the criterion applied) but is subject to change which is quantitatively expressed. Even in the latter respect, however, it may show considerable stability in certain genetic backgrounds, as attested by the W23 data presented.

SUMMARY

The present investigation was designed to test whether Modulator, a transposable component of the variegated pericarp allele P^{vv} in maize, is stable during transpositions from the *P* locus. A series of independent *Mp* transpositions derived from a common P^{vv} allele were studied in two highly uniform inbred backgrounds, 4Co63 and W23. The transposed Modulators were compared for their effect in reducing the frequency of somatic mutation of P^{vv} to P^{rr}, variegated pericarp to self-color.

Among the eight transpositions studied in the 4Co63 background the frequency of somatic mutation differed significantly. The eight transpositions fell into two apparently homogeneous groups, the inter-group differences being highly significant. This result is interpreted as indicating the existence of transposed Modulator in two different states, differing in ability to reduce the frequency of somatic mutation of P^{vv} to P^{rr}. No such difference was recognizable in terms of time of mutation during development, although the meaning of the data bearing on this point is not entirely clear.

In contrast to the above findings, the five transpositions studied in the W23 background yielded values which were not significantly different either in re-

gard to the frequency of mutation and/or distribution of mutations in the different size-classes. Thus, in this background, the stability of Modulator was uniformly maintained.

ACKNOWLEDGMENTS

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COMPLEMENTARY GENES CONTROLLING HOMOTHALLISM IN SACCHAROMYCES

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IT was demonstrated by TAKAHASHI, SAITO, and IKEDA (1958) that the hybrid between a homothallic and a heterothallic strain of Saccharomyces produced ascospores in a ratio of two diploids to two haploids, and that the obligatory diploidization of the homothallic strain might be under the control of gene *HO*. The gene *HO* was presumed to work just like the gene *D* of WINGE and ROBERTS (1949) in *Saccharomyces chevalieri*.

In these investigations, one haploid segregant from the hybrid between a homothallic and a heterothallic haploid strain was used. When the zygotes thus produced were tested by tetrad analysis, the segregation ratio with regard to the homothallic characters was 1:3. This result was taken as evidence that more than two genes are working in a complementary way for the expression of homothallic characters. When the term "homothallic" is used in this paper, it refers to a strain equipped with the characters of (1) obligatory diploidization. (2) ascospore formation, and (3) absence of mating reaction, although the mating potency was detected when tested by a minimal plate mating method (TAKAHASHI, SAITO, and IKEDA 1958).

MATERIALS AND METHODS

Among the three haploid strains referred to below, one (328–32) is an offspring of the hybrid H-328 produced from the homothallic strain S-341 and the heterothallic strain H-315, and two (L-8256 and L-17807) are the Carbondale heterothallic strains (through the courtesy of DR. C. C. LINDEGREN).

328–32 is characterized as mating type *a;* adenine requiring; galactose and sucrose nonfermenter.

L-8256 is characterized as mating type *a;* adenine requiring; galactose, sucrose, and maltose nonfermenter.

L-17807 is characterized by mating type α; arginine requiring; maltose nonfermenter.

Media: The compositions of complete medium, minimal medium and sporulation medium were given in a previous paper (TAKAHASHI, SAITO, and IKEDA 1958).

Procedures: For making crosses, LEUPOLD and HOTTINGUER'S method (1954) was modified a little. One loopful of each of two haploid strains was taken and the two were inoculated together in 2 ml of complete medium in a small test tube, and the cells were transferred every 24 hours to new complete medium. By this

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process, the test tube after 4–7 days at 25°C was filled with zygotes produced by the two testing strains.

Mating types were determined by LINDEGREN's mass mating method (LINDEGREN and LINDEGREN 1943). Homothallic segregants were judged by the negative mating reaction in the mass mating method and by positive sporulation on sporulation medium.

The nutritional requirements of the segregants were determined by the presence or absence of positive growth on minimal medium supplemented with adenine (20 mg per 1,000 ml) and/or L-arginine (100 mg per 1,000 ml). Sugar fermentation was tested in B. C. P. indicator medium (HAWTHORNE 1955).

Gene symbols: Genotypes are to be expressed in the following way: mating type (a/α); independence/dependence of adenine *(AD/ad)* and arginine *(AR/ar)*; ability/inability to ferment galactose *(GA/ga)*, sucrose *(SU/su)*, and maltose *(MA/ma)*. The homothallism controlling complimentary genes which are to be discussed in this paper will be expressed as HM_1 and HM_2 (the alleles hm_1 and hm_2).

RESULTS

In the tetrads obtained from H-675, a hybrid between L-17807 (α *AD ar GA SU ma*) and 328–32 *(a ad AR ga su MA)*, every trait except mating type showed a 2:2 segregation. With regard to mating-type, there were two classes. The segregants in the first class showed a positive mating reaction and produced no ascospores. On the other hand, the segregants in the second class mated with neither *a* nor α type strains and produced ascospores by themselves. In addition, according to the tetrad analysis carried out with some strains of this class, the genome of those tested strains were homozygous with regard to every genetic marker. Accordingly, the strains in the latter class were inferred to be homothallic diploid. When judged on these criteria, the segregation pattern of homothallic and heterothallic characters from the hybrid was 1:3 (Table 1). Because the parental strains, L-17807 and 328–32, were haploid and the hybrid between them produced homothallic diploid strains, it was supposed that each parental strain was equipped with one of the complementary genes which was able to result in homothallic diploid offspring when crossed.

On this basis, the following two genes were postulated.

(1) Gene HM_1: a complementary gene existing in the Carbondale heterothallic haploid strain L-17807.

(2) Gene HM_2: another complementary gene existing in the haploid strain 328–32 and originating from the homothallic strain S-341.

If we suppose that the hypothesis stated above is correct, the genotypes in the tetrad giving the segregation ratio 1 homothallic to 3 heterothallic must be $\frac{HM_1\ HM_2}{HM_1\ HM_2} : HM_1\ hm_2 : hm_1\ HM_2 : hm_1\ hm_2$. This expectation was proved by the following results.

From an ascus (675–1) which showed a 1:3 segregation, the three haploid spore cultures (675–11, 675–12, and 675–14) were established and crossed with

TABLE 1

Segregation data of H-675

Segregant	Genotype	Sporulation
675–11	*a AD AR ga su MA*	—
12	—* *AD ar ga su ma*	+
13	*a ad ar GA SU MA*	—
14	*a ad AR GA SU ma*	—
675–21	— *ad ar GA su MA*	+
22	*a AD AR ga SU ma*	—
23	*a AD AR GA su ma*	—
24	*a ad ar ga SU MA*	—
675–41	— *AD ar GA su MA*	+
42	*a ad AR GA SU ma*	—
43	*a ad AR ga su MA*	—
44	*a AD ar ga SU ma*	—
675–61	— *ad ar ga su MA*	+
62	*a AD AR ga SU MA*	—
63	*a ad ar GA su ma*	—
64	(nongerminating)	
675–81	*a ad ar ga SU ma*	—
82	*a ad AR ga su ma*	—
83	*a AD ar GA SU MA*	—
84	(nongerminating)	

* Negative reaction upon mass mating.

each other or with the standard strains as indicated in the footnote of Figure 1. As seen in Figure 1 and Table 2, homothallic segregants were obtained only from H-718 and H-732 and not from H-717 and H-733.

Strain 675–12 was a homothallic segregant, so its genotype is assumed to be $\frac{HM_1\ HM_2}{HM_1\ HM_2}$. Because 675–14 gave homothallic offspring when crossed with L-17807, the genotype is assumed to be $hm_1\ HM_2$. Moreover, 675–13 gave homothallic diploids when crossed with 675–14. Therefore its genotype may be the same as L-17807; that is, $HM_1\ hm_2$. Accordingly, the remaining 675–11 may be $hm_1\ hm_2$.

To test the hypothesis more extensively, the spore cultures from one ascus, 732–7 of the hybrid between L-17807 and 675–14, were intercrossed and backcrossed. As the results in Figure 2 and Table 3 show, no homothallic segregants were obtained from H-751. Therefore the genotype of 732–71 could be $hm_1\ hm_2$ or $HM_1\ hm_2$. Among the segregants from H-753, homothallic diploids were recovered, so 732–74 is $HM_1\ hm_2$. By the same reasoning, 732–73 is $hm_1\ HM_2$, and since 732–72 was a homothallic diploid, $\frac{HM_1\ HM_2}{HM_1\ HM_2}$, then 732–71 must be $hm_1\ hm_2$.

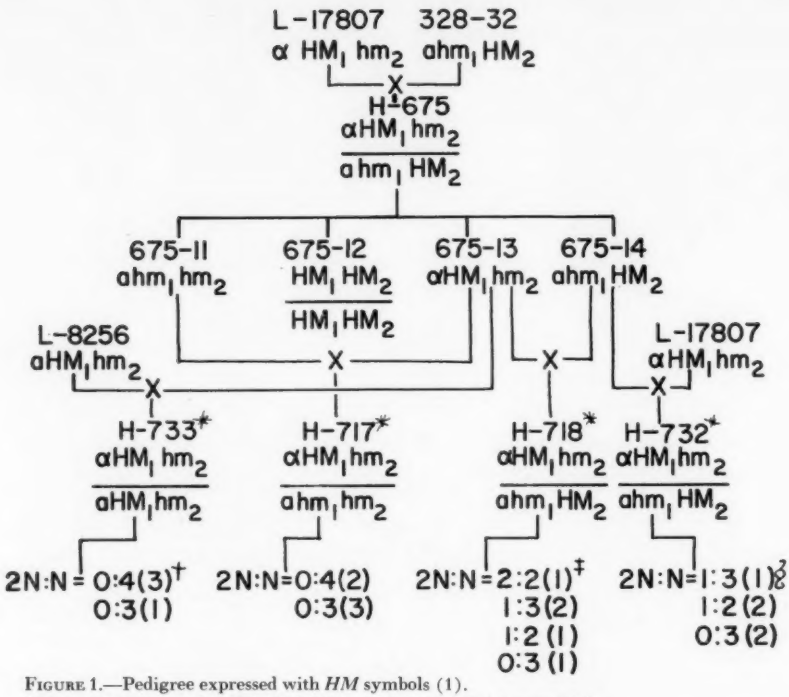

FIGURE 1.—Pedigree expressed with *HM* symbols (1).

* H-717: 675–13(*α ad ar GA SU MA*) × 675–11(*α AD AR ga su MA*)
H-718: 675–13(*α ad ar GA SU MA*) × 675–14(*α ad AR GA SU ma*)
H-732: L-17807(*α AD ar GA SU ma*) × 675–14(*α ad AR GA SU ma*)
H-733: 675–13(*α ad ar GA SU MA*) × L-8256(*α ad AR ga su ma*)

† Equation means ratio diploid to haploid segregants in ascus; the numbers of dissected asci are shown in parentheses.

‡ Genotypes of segregants are presumed to be

$$2\left(\frac{HM_1\,HM_2}{HM_1\,HM_2}\right) : 2(hm_1\,hm_2).$$

§ $1\left(\frac{HM_1\,HM_2}{HM_1\,HM_2}\right) : 1(HM_1\,hm_2) : 1(hm_1\,HM_2) : 1(hm_1\,hm_2)$.

DISCUSSION

To explain the origin of diploid segregants among tetrads expected to be haploid, supernumerary mitosis (WINGE and ROBERTS 1953) or polyploid segregation (ROMAN, PHILLIPS, and SANDS 1955) might be considered. However, it is likely that the complementary gene system concerns the present case, because (1) the segregation of *HM* genes as well as other genes was always 2:2, and (2) the diploid segregants were invariably homozygous with regard to every tested marker.

When 1:3 and 0:4 in addition to 2:2 segregations are found in the tetrad analysis of *Saccharomyces*, it is usual to suppose that complementary genes are present (EMERSON 1956). In the present experiments, the segregation ratio, homothallic:heterothallic, diverged frequently from the normal ratio of 2:2. For example, among complete asci two showed a ratio of 2:2 and seven a ratio of 1:3; among incomplete asci, two were 2:1, six were 1:2, and four were 0:3. For this reason, the author postulates complementary genes, HM_1 HM_2, which are working together for the expression of the homothallic character; i.e., those equipped with $\frac{HM_1\ HM_2}{HM_1\ HM_2}$ are homothallic diploids and those lacking either or both HM_1 or HM_2 are heterothallic haploids.

The linkage relationship betwen the mating-type genes and the homothallism controlling genes are inferable. Provided that HM_1 *and* HM_2 were not linked, the hybrid HM_1 $hm_2 \times hm_1$ HM_2 should give segregants in a ratio of parental ditype (0:4) : nonparental ditype (2:2) : tetratype (1:3) of 1:1:4. The ratio found in this work was 0:2:7 (chi-square = 1.83; $0.1 < P < 0.2$). Besides, the ratio of homothallic segregants : heterothallic segregants were 21:51, not far from the theoretical ratio of 1:3 (chi-square = 0.69; $0.3 < P < 0.5$), so that HM_1 and HM_2 may not be in the same linkage group. The hybrid between *a* HM_1 $hm_2 \times$ *a* hm_1 HM_2 gave homothallic segregants, and among them, 11 clones had

TABLE 2

Segregation data of H-718 and H-732

H-718			H-732		
Segregant	Genotype	Sporulation	Segregant	Genotype	Sporulation
718–21	— *ar ma*	+	732–21	*a ad AR*	—
22	*a AR MA*	—	22	*a AD AR*	—
23	*a ar MA*	—	23	*a ad ar*	—
24	*a AR ma*	—	24	(nongermi.)	
718–31	— *ar ma*	+	732–31	— *AD AR*	+
32	*a AR ma*	—	32	*a ad ar*	—
33	*a ar MA*	—	33	*a ad ar*	—
34	*a AR MA*	—	34	(nongermi.)	
718–41	— *ar ma*	+	732–41	*a AD ar*	—
42	*a ar ma*	—	42	*a ad AR*	—
43	*a AR MA*	—	43	*a AD ar*	—
44	(nongermi.)		44	(nongermi.)	
718–71	*a AR MA*	—	732–61	*a ad AR*	—
72	*a ar MA*	—	62	— *AD ar*	+
73	*a ar ma*	—	63	*a ad AR*	—
74	(nongermi.)		64	(nongermi.)	
718–91	*a ar MA*	—	732–71	*a ad AR*	—
92	— *AR ma*	+	72	— *AD AR*	+
93	— *AR ma*	+	73	*a AD ar*	—
94	*a ar MA*	—	74	*a ad ar*	—

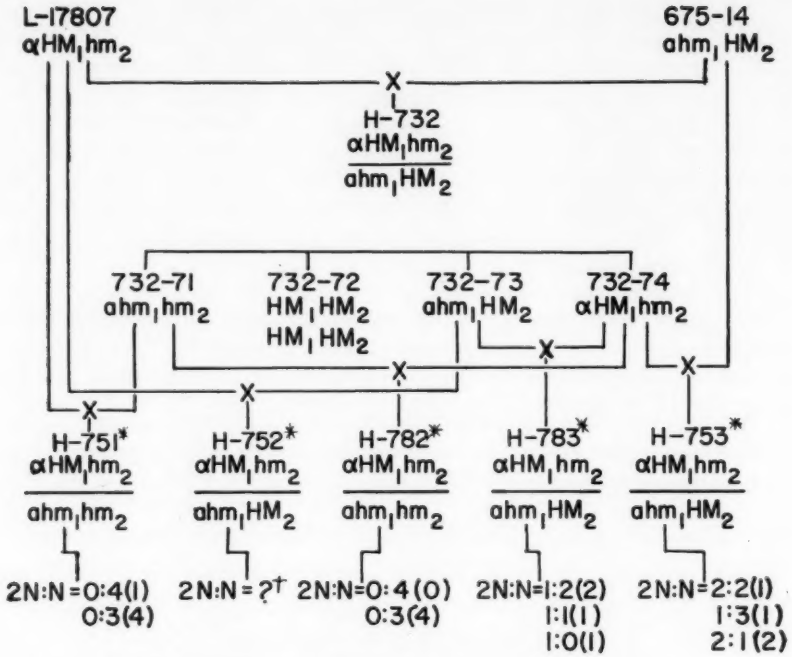

FIGURE 2.—Pedigree expressed with *HM* symbols (2).
* H-751: L-17807 (*a AD ar GA SU ma*) × 732–71 (*a ad AR GA SU ma*)
H-752: L-17807 (*a AD ar GA SU ma*) × 732–73 (*a AD ar GA SU ma*)
H-753: 732–74 (*a ad ar GA SU ma*) × 675–14 (*a ad AR GA SU ma*)
H-782: 732–74 (*a ad ar GA SU ma*) × 732–71 (*a ad AR GA SU ma*)
H-783: 732–74 (*a ad ar GA SU ma*) × 732–73 (*a AD ar GA SU ma*)
† Four-spored asci did not appear.

$\frac{a\,HM_1\,HM_2}{a\,HM_1\,HM_2}$ structure and one was $\frac{a\,HM_1\,HM_2}{a\,HM_1\,HM_2}$ (theoretical ratio: 1:1; chi-square = 8.32; $P < 0.01$). Judging from this segregation pattern, the mating type locus and the HM_1 locus may be closely linked.

As stated in the previous work with the asci of H-328, all tetrads of eight analyzed asci gave two diploid to two haploid segregation (TAKAHASHI, SAITO, and IKEDA 1958). If the two diploid segregants had been affected by the two complementary genes postulated above, the remaining two haploid segregants would have neither of the genes. Nevertheless, one of such haploid segregants, 328–32, had a complementary gene controlling homothallism (HM_2). Therefore, it is supposed that another gene HM_3 concerns the homothallic controlling system in the previous paper. Hereupon, the genotypes of tested strains may be summarized as shown in Figure 3. Namely, the original homothallic strain S-341 was

$\frac{hm_1\ HM_2\ HM_3}{hm_1\ HM_2\ HM_3}$ and the heterozygous diploid H-315 derived from the Carbondale strains, L-8256 and L-17807, was $\frac{HM_1\ hm_2\ hm_3}{HM_1\ hm_2\ hm_3}$. From crosses between S-341 and H-315 and between their offspring, two homothallic diploid strains having the genotype $\frac{HM_1\ HM_2\ hm_3}{HM_1\ HM_2\ hm_3}$ (675–12 and 732–72) and several heterothallic haploid strains having the genotypes $hm_1\ HM_2\ hm_3$ (675–14 and 732–73) or $hm_1\ hm_2\ hm_3$ (675–11 and 732–71) were recovered. If we suppose that the hypothesis described above is correct, eight kinds of gene constitutions are possible (Table 4), but among them, three gene constitutions, $\frac{HM_1\ HM_2\ HM_3}{HM_1\ HM_2\ HM_3}$, $\frac{HM_1\ hm_2\ HM_3}{HM_1\ hm_2\ HM_3}$, and $\frac{hm_1\ hm_2\ HM_3}{hm_1\ hm_2\ HM_3}$, have not been recovered.

Now, let us again examine the two diploid to two haploid segregation in the previous paper on the basis of the above three gene hypothesis.

(1) Suppose the homothallic character is controlled by the co-existence of two or three dominant genes among three alleles, it is expected that the hybrid between the homothallic strain $\frac{hm_1\ HM_2\ HM_3}{hm_1\ HM_2\ HM_3}$ and the heterothallic strain $\frac{HM_1\ hm_2\ hm_3}{HM_1\ hm_2\ hm_3}$ segregates in the following way:

homothallic : heterothallic = 3:1 (11.1 percent)

TABLE 3

Segregation data of H-753 and H-783

H-753			H-783		
Segregant	Genotype	Sporulation	Segregant	Genotype	Sporulation
753–21	*a AR*	—	783–21	*a ad*	—
22	— *AR*	+	22	— *ad*	+
23	— *ar*	+	23	*a AD*	—
24	(nongermi.)		24	(nongermi.)	
753–31	*a ar*	—	783–41	*a ad*	—
32	— *AR*	+	42	— *AD*	+
33	— *ar*	+	43	*a ad*	—
34	(nongermi.)		44	(nongermi.)	
753–41	*a AR*	—	783–111	— *AD*	+
42	— *AR*	+	112	*a AD*	—
43	*a ar*	—	113	(nongermi.)	
44	— *ar*	+	114	(nongermi.)	
753–51	*a ar*	—	783–121	— *ad*	+
52	— *AR*	+	122	(nongermi.)	
53	*a ar*	—	123	(nongermi.)	
54	*a AR*	—	124	(nongermi.)	

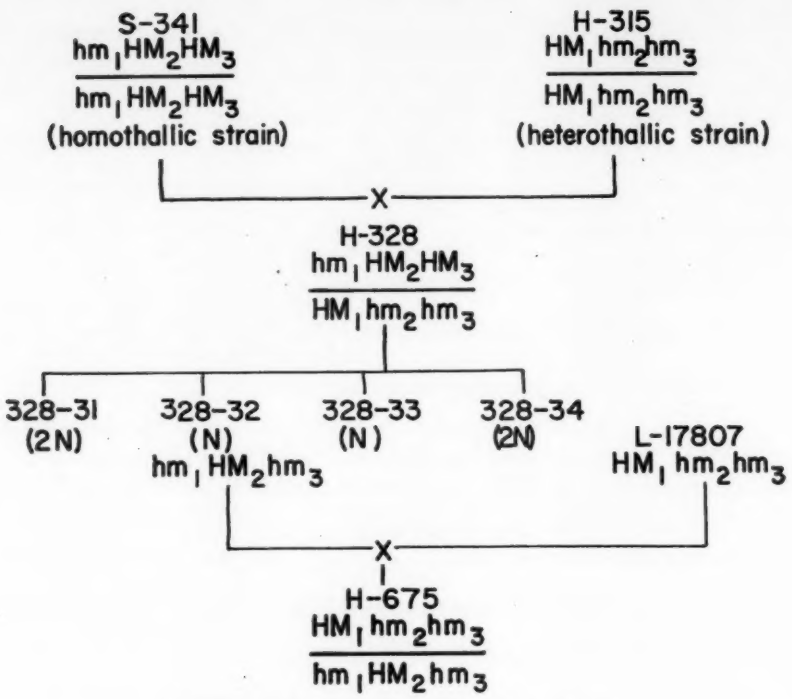

FIGURE 3.—Pedigree expressed with *HM* symbols (3).

TABLE 4

Relationship between gene constitution and the homothallic characters

Gene constitution	Mating reaction	Sporulation	Remarks
$HM_1\ HM_2HM_3$	?	?	?
$HM_1\ HM_2\ hm_3$	—	+	homothallism
$HM_1\ hm_2\ HM_3$	?	?	?
$hm_1\ HM_2\ HM_3$	—	+	homothallism
$HM_1\ hm_2\ hm_3$	+	—	heterothallism
$hm_1\ HM_2\ hm_3$	+	—	heterothallism
$hm_1\ hm_2\ HM_3$	?	?	?
$hm_1\ hm_2\ hm_3$	+	—	heterothallism

homothallic : heterothallic = 2:2 (77.8 percent)
homothallic : heterothallic = 1:3 (11.1 percent)

(2) If the diploidization of single spore cultures is induced by the existence of either the independent gene HM_3 or the complementary genes HM_1 HM_2, the theoretical ratio of phenotypic segregation is as follows:

homothallic : heterothallic = 4:0 (2.8 percent)
homothallic : heterothallic = 3:1 (41.7 percent)
homothallic : heterothallic = 2:2 (55.5 percent).

The "chi-square" value between the above two theoretical ratios and the observed ratio are 2.32 ($0.2 < P < 0.3$) in the case of (1) and 8.09 ($0.01 < P < 0.02$) in the case of (2). Therefore, the first hypothesis is more likely. As a consequence, the gene symbol *HO* employed in the previous paper as being responsible for the obligatory diploidization of spore culture might not be a single gene, but rather a phenotype may have resulted from the complementary genes. Further studies on this problem are now being undertaken.

Among the Carbondale strains, the presence of the gene HM_1 in L–8256 has not been clarified in this paper. But as the original hybrid H–328 was produced through the processes shown in Figure 4, the gene HM_1 in H–328 might have been derived from strain L–8256. Moreover, as the ascospore which united with the homothallic strain S–341 was of mating type α, which is linked with the gene hm_1 as described above, and the resulting hybrid H–328 has gene HM_1 in its genome, it may fairly be said that L–8256 which is mating type a retains the HM_1 gene in a concealed form.

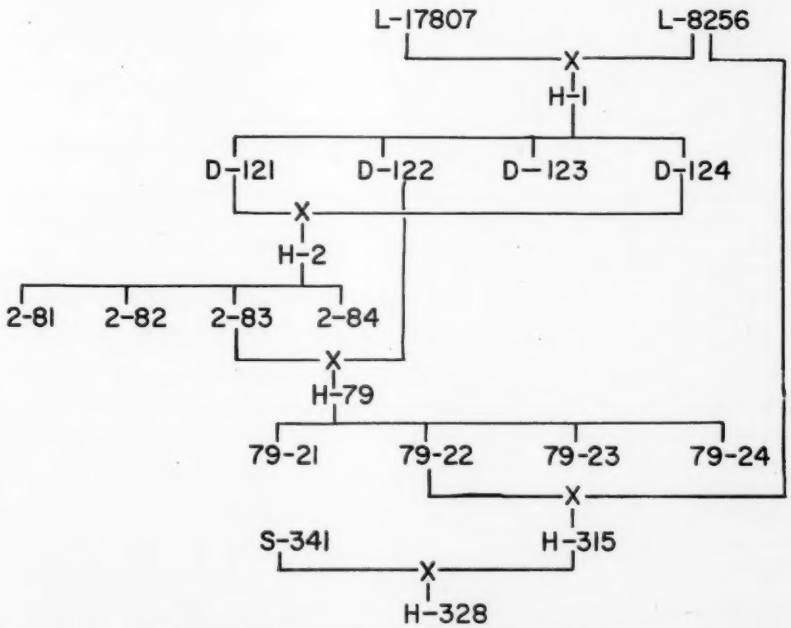

FIGURE 4.—Pedigree showing the ancestors of H-328.

SUMMARY

From the cross between a Carbondale heterothallic strain and a haploid segregant which had arisen from a hybrid between a homothallic and a heterothallic strain of Saccharomyces, zygotes giving the segregation ratio one diploid to three haploid were frequently recovered. To explain this irregular segregation, a set of complementary genes (HM_1 and HM_2) was postulated for the expression of the homothallic character (obligatory diploidization, positive sporulation, and absence of mating reaction). This hypothesis was evidenced by the cross tests between the progenies of the above mentioned zygotes and the Carbondale haploid strain. Due to the results it was concluded that the gene HM_1 did come from the Carbondale strains and the gene HM_2 from the homothallic strains.

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A COMPARISON OF THE RELATIVE EFFECTIVENESS OF RADIATIONS OF DIFFERENT AVERAGE LINEAR ENERGY TRANSFER ON THE INDUCTION OF DOMINANT AND RECESSIVE LETHALS IN DROSOPHILA

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SINCE 1927 numerous investigators have subjected various plants and animals to radiations of different ion density to determine the relative biological effectiveness (RBE) of the different radiations. Usually it was found that the RBE increased as the linear energy transfer (LET—energy in electron volts dissipated per micron of tissue traversed by ionizing particles) or ion density of the radiation increased. In a few cases the reverse was observed and in others the biological effect was independent of the LET (for a review of these data, see ZIRKLE 1954).

Data from early Drosophila work produced conflicting opinions as to the biological effectiveness of radiations of different LET for the induction of the various genetic effects studied. It was reported that the less densely ionizing beta, gamma, and X-rays were equally effective in producing sex-linked recessive lethals (ZIMMER and TIMOFÉEFF-RESSOVSKY 1942; CATCHESIDE 1948) and translocations (MULLER 1940), and more effective than the more densely ionizing fast neutrons for the induction of recessive lethals (DEMPSTER 1941; GILES 1943; TIMOFÉEFF-RESSOVSKY and ZIMMER 1938; FANO 1944) and translocations (CATSCH *et al.* 1944).

On the other hand, it was reported that fast neutrons were more effective than the less densely ionizing radiations in the production of translocations (DEMPSTER 1941) and dominant lethals (DEMPSTER 1941; FANO 1944). ZIRKLE (1954), reporting the data of STAPLETON and ZIRKLE (1946) and ZIRKLE and PARRISH (1950), however, states that the RBE for the survival of Drosophila eggs after exposure to radiations that varied in average LET increased with increasing LET except that the most densely ionizing fast neutrons from uranium fission were less effective than cyclotron neutrons yet more effective than other radiations of lower average LET. Results of recently published data indicate that the RBE of different radiations for the induction of all genetic effects increases with increasing LET (BAKER and VON HALLE 1954; MICKEY 1954; MULLER 1954; LEWIS 1954; STONE *et al.* 1954; IVES *et al.* 1955; EDINGTON 1956). Since these differences in the RBE were explained to be the result of differences in the LET of the radia-

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tions used, it would appear that within the neutron energy range available, the RBE of neutrons of different average energy would also depend on the LET.

The purpose of this paper is to compare the RBE of fast neutrons of different LET spectra with radiations of lesser ion density for the induction of sex-linked recessive lethals and dominant lethals in Drosophila. These data show that the effectiveness of different radiations on the induction of the genetic effects studied depends on the LET even within the energy range of fast neutrons available for this investigation.

MATERIALS AND METHODS

Biological

For a more accurate comparison of the results reported in this paper with those of earlier work performed in this laboratory with different radiations on the induction of dominant lethals and sex-linked recessive lethals (hereafter called recessive lethals), the experimental techniques used were the same as those already described by BAKER and VON HALLE (1953) for dominant lethals and by EDINGTON (1956) for recessive lethals. These methods will be briefly described. For the detection of dominant lethals, Oregon-R males, two to four days old were exposed to several doses of fast neutrons or gamma rays and pair-mated immediately to Oregon-R virgin females. After a 24-hour mating, the males were discarded and the females were placed on a charcoal agar medium (with 250 units per ml of Squibb's Mycostatin added to prevent yeast growth) for a 24-hour egg-laying period. After 24 hours, the females were transferred to a fresh vial containing charcoal agar for an additional 24 hours, and the eggs laid in the first vial were counted. After a second 24-hour period, the proportion of eggs that had hatched was determined by a count of collapsed eggs. Similar 24 and 48-hour counts were made on the second set of eggs.

The induction of recessive lethals by 14-Mev fast neutrons was determined by the Muller-5 method (SPENCER and STERN 1948). Oregon-R males, two to four days old, were irradiated and mass mated immediately to Muller-5 virgin females. The parents were transferred to fresh culture bottles every two days for six days. Then the males were discarded, and the females were transferred to fresh culture bottles and allowed to lay for six additional days.

In all dominant and recessive lethal tests, controls were run simultaneously with the experimental series.

Physical

The physical methods employed for the X-ray, gamma-ray, and low-energy neutron irradiations have been described (EDINGTON 1956; SHEPPARD *et al.* 1957; GHORMLEY and HOCHANADEL 1951; KIRBY-SMITH and DANIELS 1953). The 14.1-Mev neutrons used in this experiment were produced by the $T(d,n)He^4$ nuclear reaction, which satisfies many desiderata for radiobiology with a neutron source. The cross section for this reaction is great enough that experiments can be performed with modest-sized positive ion accelerators; the emitted neutrons are

monoenergetic; the reaction itself is free of significant gamma ray emission (SAWYER and BURKHARDT 1955). A 250-kev Cockcroft-Walton accelerator (BERGSTRALH *et al.* 1953) with radiofrequency ion source (MOAK *et al.* 1951) produced the neutrons by bombardment of a thick zirconium tritide target on a platinum backing (MASSEY 1957) with deuterons. Figure 1 illustrates details of the target assembly and irradiation setup. Intolerable local target temperatures were avoided by a thin (0.6 mm), rapidly flowing stream of tap water and by rotation of the target about a ball and socket joint not shown in Figure 1. Deuteron beam currents

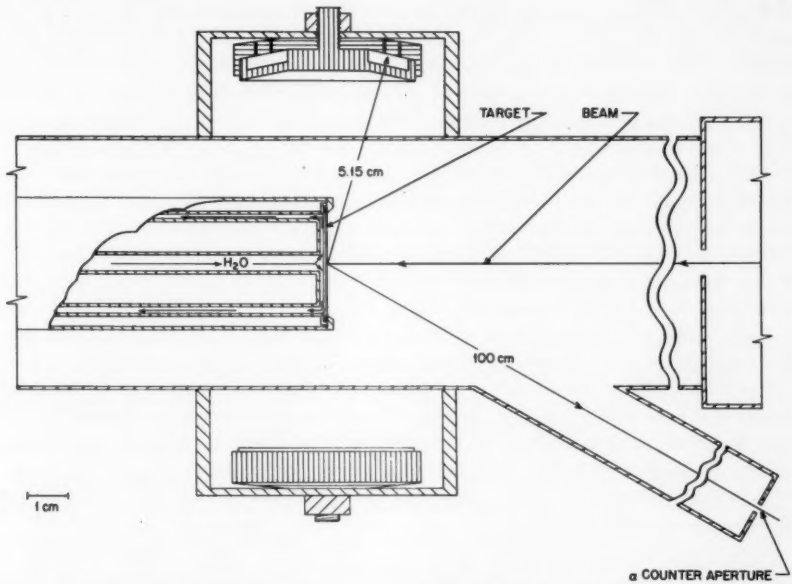

FIGURE 1.—Physical conditions for exposure of Drosophila to monochromatic 14-Mev fast neutrons.

of several hundred microamperes produced average dose rates of about 800 rep per hour at the distance of the biological material. The flies were contained in plastic cups at 4.90 to 5.30 cm from the center of the target with the average distance about 5.15 cm. Perforated lids prevented anoxia for periods well in excess of the duration of irradiation. The cups were machined from nylon, which has been calculated to be the common plastic most nearly tissue equivalent for 14-Mev neutrons (RANDOLPH 1957a), and their wall thickness of 2.5 mm insured secondary equilibrium for 14-Mev protons. From target discoloration, the over-all cross sections of the deuteron beam was roughly an equilateral triangle with 5 mm sides. Most of the ions, however, were within a central circular focal area of about 2 mm diameter. This central spot was thought to be located to within 2 mm. To

minimize the possible error caused by miscentering of the beam, half of each dose was administered with the flies in one position and half with them on the diametrically opposite side of the target. Thus the effect of a 3 mm error in centering would be reduced to less than one percent at this distance. The accelerator room was air-conditioned with forced ventilation and temperature of 20° C.

The absolute dosimetry is based on intercomparisons of several independent systems of fast neutron dosimetry. The systems included: a small, homogeneous polystyrene-acetylene ion chamber (DARDEN and SHEPPARD 1951) with the Townsend method for measurement of ionization with a vibrating reed electrometer (Applied Physics Corporation Model 30-C) as a null detector, a Hurst absolute fast neutron detector (HURST *et al.* 1954), and conversion to dose (RANDOLPH 1957a) from absolute fast neutron flux determinations obtained by: (1) a proportional counter for measurement of the companion alpha particles of the $T(d,n)He^4$ reaction and (2) a long counter with flat neutron response up to 14 Mev (GRAVES *et al.* 1955) calibrated versus a known Po-Be neutron source. Intercomparisons of these methods agreed to within ten percent, which was the same general magnitude as the expected errors in absolute values by any one system. Hence the average values were thought to be correct to within ten percent. The gamma ray contamination, which was caused by neutron interactions at the walls of the room and near the target, was determined to be less than five percent of the total dose by a special ion-chamber proportional counter, when used as an ion chamber, measured total dose; and as a proportional counter, measured only fast neutron dose by electronic discrimination against gamma radiation.

For a determination of the doses, the elemental composition of Drosophila sperm was assumed equivalent to that of wet tissue. In our experiments for 14-Mev neutrons, where most of the ionizing particles originate from nuclear interactions throughout the flies or in the nylon (tissue equivalent) cups, this assumption seems justified. The same general argument holds for the conditions of our X and gamma irradiations.

RESULTS

The data obtained in each of the radiation runs reported in this paper, with the exception of the dominant lethal experiment with gamma rays that consisted of a single experiment at each dose, were tested for homogeneity by the χ^2 method at each dose level. The variation observed in the recessive lethal tests was that expected from random sampling of binomial populations; therefore, the average value for each dose with its standard error (S.E.) was tabulated in Table 1. The dominant lethal results, however, were heterogeneous; consequently, the results of each individual experiment at each dose were listed separately in Table 2 with the gamma-ray dominant lethal data and their standard errors.

Weighted regressions that best fit the experimental data for each genetic effect studied were calculated by the least-squares method. Since there was approximately five percent gamma contamination in the total dose from the neutron exposures, a simultaneous fitting similar to that used by RUSSELL *et al.* (1954)

was performed. It included a linear term (recessive lethals) and an exponential (dominant lethals) and accounted for the five percent gamma contamination (assumed to be no different from gamma rays emitted by a Co^{60} source). The linear equation, $y = 0.11 - 2.39\ D$ (where y is the predicted percentage and D the dose in kilorep), was found to fit the fast neutron recessive lethal data. The dominant lethal frequency-dose relation for gamma rays was best described by the exponential quadratic equation, $\ln y = \ln 97.17 - 0.144\ D - 0.021\ D^2$, and an exponential linear equation, $\ln y = \ln 97.49 - 0.48\ D$ (where y is in percentage survival and D is the dose in kilorep) gave the best fit to the monochromatic 14-Mev fast neutron data. Although the χ^2 values for goodness of fit of the experimental data for both gamma ray- and fast neutron-induced dominant lethals to their respective regression were high ($P = < 0.01$), there were no systematic departures from expectation.

TABLE 1

The percentage of sex-linked recessive lethals induced by monochromatic 14-Mev fast neutrons

Dose (rep)	Chromosomes tested	Number of lethals	Percentage lethals
0	3309	10	0.30±0.09
1000	3000	71	2.37±0.28
2000	2359	136	5.77±0.48
3000	1407	101	7.18±0.69
4000	1294	105	8.11±0.76
5000	747	97	12.99±1.23

TABLE 2

The percentage of egg hatch after exposure to Co^{60} gamma rays and 14-Mev fast neutrons

Gamma rays				14-Mev fast neutrons			
Dose (rep)	Eggs laid	Eggs hatched	Percentage egg hatch	Dose (rep)	Eggs laid	Eggs hatched	Percentage egg hatch
0	1294	1272	98.30±0.36	0	1591	1533	96.35±0.47
1000	1131	947	83.73±1.10		1209	1183	97.85±0.42
2000	1018	716	70.33±1.43		1424	1390	97.61±0.41
3000	987	509	51.57±0.60	1000	1344	816	60.71±1.33
4000	1093	405	37.05±1.46		392	249	63.52±2.43
5000	1146	311	27.13±1.31		1388	902	64.99±1.24
6000	796	182	22.86±1.49	2000	1813	689	38.00±1.14
					924	379	41.02±1.62
					1592	586	36.81±1.21
				3000	1297	289	22.28±1.16
					847	184	21.72±1.42
				4000	1290	176	13.64±0.96
					627	61	9.73±1.18
					1148	131	11.41±0.94
				5000	1031	96	9.31±0.91
					234	20	8.55±1.83

In Figure 2 the weighted linear regression for recessive lethals on dose for monoenergetic 14-Mev fast neutrons is plotted with the regressions for recessive lethals on dose for Co^{60} gamma rays, X-rays, and 1-Mev fast neutrons reported by EDINGTON (1956). The regressions for percentage egg hatch (1− dominant lethals) on dose for gamma rays and 14-Mev fast neutrons are plotted in Figure 3 with the regressions of egg hatch on dose for X-rays ($\ln y = \ln 95.03 - 0.17\,D - 0.04\,D^2$) and 1-Mev fast neutrons ($\ln y = \ln 97.0 - 0.88\,D$) reported by BAKER and VON HALLE (1953) and SHEPPARD *et al.* (1957), respectively. In both Figures 2 and 3, the data have been corrected for the unirradiated controls.

From both the recessive lethal and dominant lethal results, RBE values were calculated, with Co^{60} gamma rays as a base for comparisons. The RBE of the different radiations for the induction of recessive lethals is determined simply by a comparison of the slopes of the regressions for each radiation with that for gamma rays. With X-rays, however, the nonlinear frequency versus dose relation prevents such a comparison because the RBE is dose dependent. Since the logarithmic regressions for percentage hatch on dose are curvilinear for X and gamma rays and linear for both 1- and 14-Mev fast neutrons, no RBE comparison applicable for all levels of biological damage can be made. Consequently, the RBE values were obtained by comparison of the estimated doses of each radiation corresponding to 50 percent egg hatch. The RBE values for the induction of recessive and dominant lethals are tabulated in Table 3.

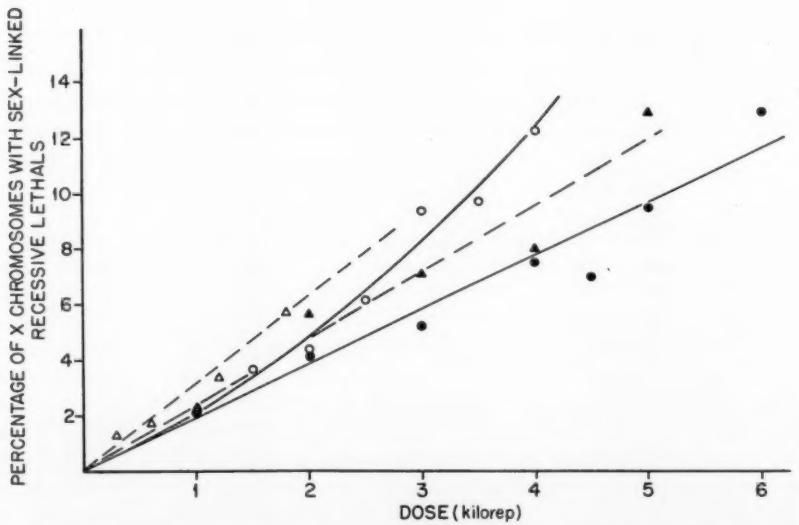

FIGURE 2.—The percentage of X chromosomes with recessive lethals induced by gamma rays (●), X-rays (○), 1-Mev (△), and 14-Mev fast neutrons (▲), in *Drosophila melanogaster*.

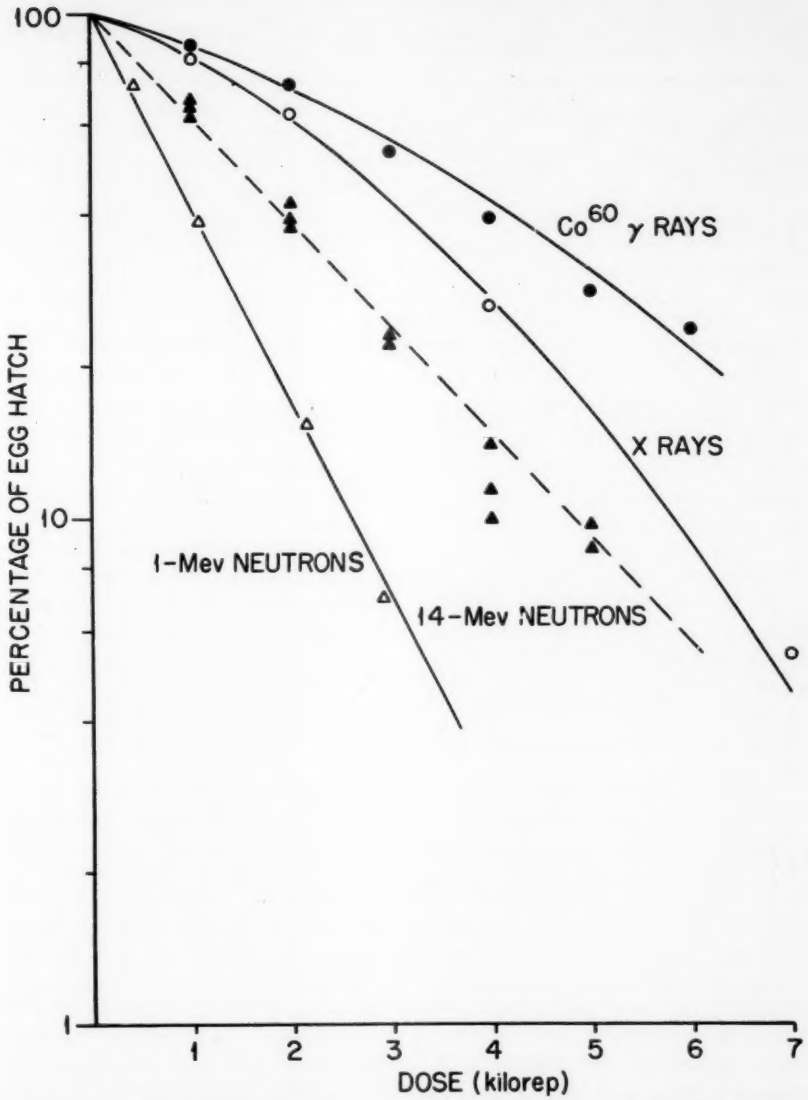

FIGURE 3.—The percentage of dominant lethals induced in sperm of Drosophila by gamma rays (●), X-rays (○), 1-Mev (△), and 14-Mev (▲) fast neutrons.

TABLE 3

The relative biological effectiveness of radiations of different average linear energy transfer on the induction of recessive lethals and dominant lethals

Radiation	LET (kev/μ)		RBE	
	Energy average	Track average	Recessive lethals	Dominant lethals (50% hatch)
Gamma rays	0.32	0.27	1.0 varies with dose	1.0
X-rays	2.2	1.8	1.1 at 1000 r; 1.4 at 4000 r	1.24
Fast neutrons				
14 Mev	75.0	12.0	1.23	2.27
1 Mev	67.0	46.0	1.62	4.23

DISCUSSION

The radiations used in the experiments discussed produce high-energy secondary ions (electrons in the photon irradiations, atomic nuclei in the neutron irradiations) that dissipating their energies by ionization and excitation, cause the biological effects observed as a result of a series of biochemical effects. The differences in effectiveness per unit dose of different ionizing radiations are thought to be caused primarily by differences in LET occuring in the irradiated material. Unfortunately, except for monoenergetic charged-particle irradiations of materials that are thin compared to the range of the particles, radiations produce a wide and complex spectrum of LET values throughout the irradiated material. Although mathematical or graphical description of LET spectra, which may be given, constitute the means for complete description of the LET spectra, the simplicity of single number characterizations of LET spectra seems more desirable for comparisons and interpretations of these studies than other more-detailed means.

Of several possible single-number, simply derived characterizations, we choose to consider only two here, although it may well be that no single number can adequately describe the LET spectra. One is what we call the "track average LET," which is that obtained when equal statistical weights are assigned to equal track lengths of all ionizing particles. This may be obtained from a division of the total energy of the ionizing particles by the total track length produced in the irradiated material. The other characterization, which we call the "energy average LET," is that average obtained when equal statistical weights are assigned to equal amounts of energy dissipated by the ionizing particles. This average is not so readily pictured as the result of elementary physical measurements of path length and energy, but might be, as BURCH and BIRD (1955) have mentioned, the natural physical average one would use. LEA (1955) and ZIRKLE (1954) used what we call the track average. BOAG (1954) has discussed both averages briefly. BURCH and BIRD (1955) and HURST *et al.* (1956) used the energy average. RANDOLPH (1957b) has given a mathematical definition of these averages.

The LET values given in Table 3 were calculated from various pieces of published data. The Co^{60} gamma-ray and the X-ray figures are based on JOHNS' review (JOHNS 1956) and the cyclotron neutron figures on an approximate estimate of the neutron spectrum by SHEPPARD *et al.* (1957). Because of differences in tube potential and filtration, the X-ray data are not strictly applicable to our work, but other uncertainties are thought to be more important. We have neglected the effect of delta rays, although BURCH and BIRD (1955) showed that this raises the energy average LET for X-rays and Co^{60} gamma rays to about 10 kev per μ and 7 kev per μ. Consideration of delta rays would reduce the track average values. We calculated our neutron LET values for first collisions in wet tissue (LEA 1955) of unit density surrounded by enough wet tissue to give secondary equilibrium. We started from BOAG's (1954) distribution functions, extended his work to include heavy recoils with the help of SNYDER and NEUFELD's evaluation (SNYDER and NEUFELD 1957) of stopping powers at low velocities near and below those at the Bragg peak of ionization, and, particularly for the 14-Mev neutrons, took into account the inelastic nuclear collision processes and angular distribution of elastically scattered neutrons (RANDOLPH 1957a). That the energy average is greater than the track average is most evident for the 14-Mev neutrons, in which 70 percent of the dose is contributed by recoil protons with energy average LET of about 15 kev per μ and 30 percent heavy particles with energy average LET of more than 200 kev per μ.

Throughout our LET calculations we have assumed that the number of electrons per unit volume of the biological material is negligibly different from that for water; whereas in these experiments the volumes of biological interest with high concentrations of nucleoproteins probably have 20 to 30 percent more electrons per unit volume. This consideration tends to increase all LET values by about the same factor, which has not been included in Table 3.

From comparisons of results of these investigations with those previously published from this laboratory, it is obvious that the relative effectiveness of the different radiations on the induction of the genetic effects studied is dependent on the average LET of the radiation used. If the track average is accepted as the proper representation of the average LET, then the RBE increases as the LET increases even within the neutron energy range studied. If, on the other hand, the energy average is the best estimate of the average LET, the RBE increases, passes through a maximum, and then decreases with increasing LET. This is true for both the recessive lethal and dominant lethal data; however, for recessive lethals the RBE-LET relation is not so clear cut because of the nonlinear frequency-dose relation for X-rays. Regardless of the method used for the determination of the average LET values, it is evident that the present RBE data is markedly different from the reported results of earlier investigations, especially for the induction of recessive lethals by radiations of different specific ion density.

Examination of the recessive lethal data reveals a close similarity in the relative effectiveness of X-rays and monochromatic 14-Mev fast neutrons. This observation is of especial interest when one attempts to explain the discrepancy between

the present RBE results in Drosophila and those of the earlier genetic investigators. The results obtained by TIMOFÉEFF-RESSOVSKY and ZIMMER (1938) and ZIMMER and TIMOFÉEFF-RESSOVSKY (1938, 1942) will be used here for comparative purposes since the only adequate description in the early literature of the physical conditions of exposure was given in these publications.

SHEPPARD *et al.* (1957) have already pointed out that the average energy of the fast neutrons in the experiments of TIMOFÉEFF-RESSOVSKY and ZIMMER was probably much higher than the 3.9 Mev reported. Since this is true, the early neutron results should approach more closely the results obtained with 14-Mev instead of 1-Mev fast neutrons. Although there is some uncertainty about whether the flies were surrounded by sufficient tissue equivalent material in the early neutron experiments, we think it unlikely that the entire discrepancy observed in the RBE values of the present and earlier experiments is caused by differences in neutron dosimetry. It is, therefore, necessary that the biological techniques used be examined more closely. Although the mating techniques of the experiments are not entirely dissimilar, ZIMMER and TIMOFÉEFF-RESSOVSKY (1938) stated in the biological procedures of their neutron series, "Bestrahlt wurden 2 bis 3 Tage alt Drosophila melanogaster ♂ ♂, die etwa 48 Stunde nach Bestrahlung mit ClB ♀ ♀ gekreuzt wurden." This statement may be the answer to the problem at hand. It is now known from the work of BAKER and VON HALLE (1953) that the frequency of detectable genetic damage is significantly lower in the offspring of X-irradiated males that have been held with or without females for 24 or 48 hours. Although this is not true when fast neutrons of 1-Mev average energy are used (BAKER and VON HALLE 1954), unpublished evidence obtained in this laboratory shows that 14-Mev fast neutrons that have an average energy more nearly equivalent to the neutrons used by ZIMMER and TIMOFÉEFF-RESSOVSKY, behave in this respect as X-rays. Therefore, the biological results of the fast neutron irradiations of ZIMMER and TIMOFÉEFF-RESSOVSKY would be lower than if the males were mated immediately after exposure. If the fast neutron data of ZIMMER and TIMOFÉEFF-RESSOVSKY were compared with X-ray results observed from immediate mating after exposure, the resulting RBE would be erroneously high. If this were true, a corrected RBE of fast neutrons determined from the data of ZIMMER and TIMOFÉEFF-RESSOVSKY would be similar to the RBE reported by us.

It is interesting that in almost all investigations in which radiations of different average LET were compared for a determination of their relative effectiveness for the induction of dominant and recessive lethals, the RBE for the induction of dominant lethals (that almost certainly originate from chromosome breaks) for any given radiation was greater than that for recessive lethals. The values obtained in these experiments also indicate the RBE for dominant lethals is higher than that for recessive lethals and, as the average LET of the radiation increases, the discrepancy between the RBE for dominant lethal and recessive lethal induction becomes greater. This difference could be caused by increased dominant lethality induced by the more effective radiations, which would in turn preferentially siphon off potential recessive lethals that would arise as the result of

break dependent deficiencies or chromosome aberrations. It is also possible that the different genetic events (point mutations, deficiencies, and lethals associated with chromosome changes) that express themselves as recessive lethals have different RBE values and the RBE observed for total recessive lethals is an average of the individual values.

SUMMARY

An investigation was made of the effects of monochromatic 14-Mev fast neutrons and Co^{60} gamma rays on the induction of dominant lethals and of 14-Mev neutrons on the production of sex-linked recessive lethals in Drosophila. These results were compared with those of other reports from this laboratory, in which radiations of different average LET were used. It was shown that the RBE of different radiations for the induction of both genetic effects studied is dependent on the LET of the radiation used.

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COMPARISON OF EFFECTS OF X-RAYS AND THERMAL NEUTRONS ON DORMANT SEEDS OF BARLEY, MAIZE, MUSTARD, AND SAFFLOWER[1]

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COMPARISONS of the biological effects of X-rays and thermal neutrons on dormant seeds of barley have been made by seedling height studies, root tip cytological analyses, and cytological studies of pollen mother cells in the first generation following irradiation and by seedling mutation studies the second generation after irradiation. The results of these studies have been reported by CALDECOTT, FROLIK, and MORRIS (1952) and CALDECOTT, BEARD, and GARDNER (1954). The objective of the research reported in this paper was to extend the comparison of these two types of radiation by studying their effects on dormant seeds of four widely different crop species: barley (*Hordeum vulgare* L.), maize (*Zea mays* L.), mustard (*Brassica juncea* Coss), and safflower (*Carthamus tinctorius* L.).

That survival or death of individual cells in an irradiated organism is dependent solely upon the nature and extent of the induced chromosomal changes has been postulated by various workers (SAX 1942; LEA 1947). The behavior of these individual cells is in turn thought to be reflected in the growth and survival to maturity of plants grown from irradiated seeds. In a study such as the one reported here, the use of four species which vary greatly in sensitivity to radiations as well as in many other characteristics should indicate whether or not the large differences in sensitivity are due to an inherent resistance of the chromosomes to radiation damage or to some other factor(s).

MATERIALS AND METHODS

Seeds of barley (var. Himalaya), maize (Single cross L289 × I205), mustard (var. Zellar's), and safflower (var. Nebraska 852) were irradiated with X-rays and thermal neutrons at the Brookhaven National Laboratory using the procedure described by CALDECOTT *et al.* (1954). Ten 500-seed samples of each species were subjected to irradiation and an eleventh 500-seed sample served as the non-treated control. The irradiation treatments consisted of five levels of

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X-rays and five of thermal neutrons for each species, with one 500-seed sample being used per treatment. As indicated in Tables 1, 2, 3, and 4, different dosage levels were used for the four species. The dose ranges were based upon preliminary experiments in which plant height was used as an indication of the relative sensitivities of the species studied.

Plant height and survival studies

For each species, 100 seeds from each of the 11 lots were planted in greenhouse flats. Seeds were spaced approximately one inch apart in rows spaced three inches apart. Determinations of plant height and survival were made at two or more times after planting for barley, maize, and mustard. For safflower, determinations of height and survival were recorded at only one age. All plant height measurements were made by holding the leaves erect and measuring the highest point of the highest leaf.

Pollen studies

Samples consisting of 250 seeds from each treatment of maize and mustard were planted in the field at Lincoln, Nebraska, in the spring of 1953. Samples consisting of 150 seeds from each treatment of barley and safflower were planted at each of two locations, Scottsbluff, Nebraska, and Fort Collins, Colorado (also in the spring of 1953). Pollen samples were collected at a stage when the pollen was stainable but before the anthers had dehisced, one sample being taken from each plant in each treatment. A 2- to 3-inch portion of the central spike of the tassel was collected from each maize plant, a single spike from each barley plant, a cluster of flowers from each mustard plant, and a single head (inflorescence) from each safflower plant. There was ample material in each individual sample for several separate determinations of pollen abnormality. Fresh samples were killed in Carnoy's solution (six parts 95 percent ethyl alcohol, three parts chloroform, and one part acetic acid) and stored at 0° F until analyzed.

The method used to determine percentage of normal pollen was similar for all species. Pollen was stripped from an anther in a drop of acetocarmine, debris was removed, and the drop was stirred to obtain an even distribution of normal and abnormal pollen grains. A cover slip was placed on the slide and counting was done with the aid of a microscope and a Micam camera with the ground glass screen in position. The 10× objective lens was used for all observations. The 25× ocular lens was used for observations of mustard and safflower pollen, and 10× and 15× oculars were used for corn and barley, respectively. Pollen grains which stained uniformly dark throughout were counted as normal, unless they were extremely small for the species concerned. All other grains, and the small pollen grains, were classified as abnormal.

A preliminary survey was made to determine the most efficient method of estimating the mean percentage of normal pollen from maize plants in the various treatments. The procedure was similar to that used by PITTENGER (1951). For each treatment in the study, ten plants, three flowers from each plant, and two anthers from each flower, all chosen at random, were used. After pollen from

each individual anther had been evenly distributed on a slide, counts of normal and abnormal pollen were made on three strips across the slide. Analysis of variance technique was then used to estimate the various components of variation contributing to "error" in estimating treatment means. The component of variation resulting from differences among plants was found to be the most important single factor in determining the variance of a treatment mean. The components of variance attributable to differences among flowers from the same plant, among anthers from the same flower, and among strips counted for the same anther were relatively small in most treatments. Hence the most efficient method of sampling to estimate the percentage of normal pollen for each treatment was to sample several plants per treatment using a single anther from each plant and counting a single strip on the slide. This also proved to be an efficient method of estimating the percentage of normal pollen for different treatments of barley, mustard, and safflower. Whenever possible, a sufficient number of plants was used to reduce the standard error of each estimate to approximately three percent.

RESULTS AND DISCUSSION

Results of the survival, height, and pollen studies are shown for barley, maize, mustard, and safflower in Tables 1, 2, 3, and 4, respectively. Correlation coefficients for the relationships between dose of X-rays or thermal neutrons and the various criteria of irradiation effect are presented in Table 5.

The preponderance of high correlation coefficients in Table 5 indicates that in the species tested, plant height, survival, and the frequency of normal pollen are, in most instances, reliable criteria of X-ray and thermal neutron effects. However, there are important exceptions. The need for care in interpreting plant height and

TABLE 1

Height, survival, and frequency of normal pollen in barley plants[1] from control and irradiated seeds

Treatment of seeds	Height (centimeters) Mean±S.E.		Survival (plants/100 seeds)		Normal pollen (percentage) Mean±S.E.
	11 days	32 days	11 days	32 days	
Control	18.5±0.18	65.1±0.67	98	98	78.3±1.5
X-rays					
8,000r	11.1±0.43	45.2±1.32	92	93	59.2±2.0
12,000r	7.4±0.61	38.0±2.00	69	58	62.0±2.1
16,000r	5.7±0.71	35.9±1.29	52	35	60.0±2.6
24,000r	3.1±0.63	30.9±3.41	28	14	57.3±4.3
32,000r	2.0±0.39	23.2±3.23	45	18	45.5±6.7
Thermal neutrons					
0.32×10^{13} N_{th}/cm^2	15.2±0.25	57.6±0.70	100	100	55.9±2.2
0.51×10^{13} N_{th}/cm^2	13.0±0.24	49.9±0.87	100	100	48.2±2.5
1.01×10^{13} N_{th}/cm^2	5.5±0.18	29.3±0.96	100	96	35.7±2.2
1.78×10^{13} N_{th}/cm^2	1.7±0.08	7.3±0.55	91	32	all plants died
1.80×10^{13} N_{th}/cm^2	0.9±0.08	6.5±3.91	70	3	all plants died

[1] Plants were grown in the greenhouse for height and survival studies and in the field for pollen studies.

survival data is clearly shown in the results with X-irradiated maize. In this case even plants of the highest dosage levels had relatively good survival at 12 days after planting, but at 32 days many of the plants from seed receiving higher doses had died and a striking increase in absolute magnitude of the correlation coefficient resulted. Conversely, at 12 days a close relationship existed between dose of X-rays and plant height, but at 32 days this relationship was much less distinct.

TABLE 2

Height, survival, and frequency of normal pollen in maize plants[1] from control and irradiated seeds

Treatment of seeds	Height (centimeters) Mean±S.E.		Survival (plants/100 seeds)		Normal pollen (percentage) Mean±S.E.
	12 days	32 days	12 days	32 days	
Control	29.7±0.41	74.9±0.93	99	99	95.9±0.3
X-rays					
4,000r	25.2±0.38	65.7±1.04	94	94	86.5±3.4
8,000r	19.0±0.43	54.9±1.12	93	93	67.6±2.8
12,000r	10.5±0.53	36.5±1.48	92	73	58.0±3.1
16,000r	5.3±0.44	28.6±2.10	87	39	62.3±4.2
24,000r	2.9±0.47	51.5±6.73	91	7	60.8±11.2
Thermal neutrons					
1.01×10^{13} N_{th}/cm^2	22.8±0.30	61.9±0.77	95	95	70.9±3.2
1.21×10^{13} N_{th}/cm^2	18.1±0.34	52.2±0.81	94	92	58.0±2.8
2.00×10^{13} N_{th}/cm^2	12.7±0.34	40.1±0.77	93	92	41.1±2.9
3.25×10^{13} N_{th}/cm^2	3.7±0.13	11.8±1.29	95	16	all plants died
3.26×10^{13} N_{th}/cm^2	3.0±0.10	12.2±4.86	95	5	all plants died

[1] Plants were grown in the greenhouse for height and survival studies and in the field for pollen studies.

TABLE 3

Height, survival, and frequency of normal pollen in mustard plants[1] from control and irradiated seeds

Treatment of seeds	Height (centimeters) Mean±S.E.		Survival (plants/100 seeds)		Normal pollen (percentage) Mean±S.E.
	15 days	32 days	15 days	32 days	
Control	6.3±0.30	23.0±1.16	41	41	93.4±1.0
X-rays					
48,000r	4.4±0.26	14.4±0.97	49	45	84.8±2.6
64,000r	3.8±0.25	13.5±0.90	46	40	63.1±3.0
80,000r	3.1±0.27	11.5±0.58	31	23	58.7±3.6
120,000r	1.6±0.08	2.8±0.58	42	9	35.8±5.2
160,000r	1.3±0.07	0.7	37	1	24.8±10.3
Thermal neutrons					
3.81×10^{13} N_{th}/cm^2	5.9±0.27	19.8±0.75	48	48	78.9±2.4
4.97×10^{13} N_{th}/cm^2	5.8±0.29	19.0±0.83	45	45	60.2±2.8
7.17×10^{13} N_{th}/cm^2	5.5±0.15	16.0±0.49	59	57	58.4±3.1
8.91×10^{13} N_{th}/cm^2	4.7±0.22	13.6±0.78	47	47	49.0±3.5
10.70×10^{13} N_{th}/cm^2	3.4±0.19	10.0±0.62	42	42	22.8±3.2

[1] Plants were grown in the greenhouse for height and survival studies and in the field for pollen studies.

TABLE 4

Height, survival, and frequency of normal pollen in safflower plants[1] from control and irradiated seeds

Treatment of seeds	Height (centimeters) Mean±S.E. 19 days	Survival (plants/100 seeds) 19 days	Normal pollen (percentage) Mean±S.E.
Control	13.9±0.56	57	95.6±0.8
X-rays			
8,000r	11.1±0.83	35	82.6±3.0
12,000r	11.4±0.62	43	80.6±2.8
16,000r	8.9±0.84	24	76.8±3.2
24,000r	7.5±0.47	22	61.0±4.3
32,000r	6.0±0.54	9	56.5±5.4
Thermal neutrons			
1.78×10^{13} N_{th}/cm^2	9.9±0.65	50	53.2±4.0
1.80×10^{13} N_{th}/cm^2	11.0±0.59	53	48.9±4.0
2.57×10^{13} N_{th}/cm^2	8.0±0.67	34	39.9±5.5
3.81×10^{13} N_{th}/cm^2	4.5±0.44	37	23.0±5.5
4.97×10^{13} N_{th}/cm^2	2.3±0.29	16	12.9 (1 plant only)

[1] Plants were grown in the greenhouse for height and survival studies and in the field for pollen studies.

TABLE 5

Correlation coefficients[1] for the relationships between various criteria of irradiation effect and dose of X-rays or thermal neutrons applied to seeds of barley, maize, mustard, and safflower

Species	Age[2]	Dose and plant height X-rays	Dose and plant height Thermal neutrons	Dose and survival X-rays	Dose and survival Thermal neutrons	Dose and normal pollen X-rays	Dose and normal pollen Thermal neutrons
Barley	11	—0.936‡	—0.984‡	—0.880*	—0.730	—0.907*	*—0.960**
	32	—0.943‡	—0.997‡	—0.923‡	—0.891*		
Maize	12	—0.964‡	—0.995‡	—0.769	—0.526	—0.831*	*—0.991‡*
	32	—0.660	—0.995‡	—0.958‡	—0.895*		
Mustard	15	—0.980‡	—0.899*	—0.343	—0.212	—0.976‡	—0.961‡
	32	—0.985‡	—0.983‡	—0.911*	—0.217		
Safflower	19	—0.980‡	—0.991‡	—0.943‡	—0.921‡	—0.987‡	—0.970‡

[1] Degrees of freedom: underlined values—2, all other values—4.
[2] Days from planting to measurements of height and survival.
* Significant at the five percent level of probability.
‡ Significant at the one percent level of probability.

This change in relationship between dose and height was related to the fact that usually plants damaged most by the high doses were alive and measurable at 12 days, but many of these plants had died prior to the 32-day measurements. Thus the most appropriate time for recording survival data may differ appreciably from

the most suitable time for taking measurements of plant height. The data indicate that for many of the seedlings from X-irradiated seeds of barley, maize, and mustard and from neutron treated barley and maize seeds death occurred during the third and fourth weeks after planting. Therefore, for these species it appears desirable to record height measurements during the second week after planting and survival measurements about one month after planting.

The pollen data are subject to errors of interpretation similar to those just mentioned for plant height and survival data. Thus, in the higher treatment levels on all species, survival at the time of pollen shedding was considerably reduced, and it is probable that the plants remaining were not truly representative of the treatments. Despite this obvious weakness of pollen analysis as a measure of irradiation effect, statistically significant correlation coefficients with high predictive value were obtained for the relationship between dose and effect for all four species and both types of radiation.

To facilitate comparisons among the four species, plant height and normal pollen data for each species were expressed as percentages of their respective control values. Linear regression coefficients were calculated and lines showing the relationship between normal pollen and plant height were then plotted for each species for the two types of radiation. The regression coefficients and their standard errors are presented in Table 6 and the regression lines are shown in Figure 1 (X-rays) and Figure 2 (thermal neutrons). It is apparent that for each of the species tested, the slope of the regression line is greater for the X-ray treatments than for the neutron treatments. That is, per unit of reduction in normal pollen, plant height was reduced more in the X-ray series than in the thermal neutron series. MACKEY (1951) and CALDECOTT *et al.* (1952, 1954) postulated an "extrachromosomal" or "physiological" effect of X-rays on seeds of barley. This suggestion was based on the observation that plant height and survival were reduced less, relative to the number of chromosomal aberrations, in plants grown from seeds exposed to fast or thermal neutron radiation than in plants grown from X-irradiated seeds. Later CALDECOTT (1956, 1958) and CALDECOTT *et al.*

TABLE 6

Regression coefficients indicating the regression of plant height on percent normal pollen in four plant species whose dormant seeds were treated with varying doses of either X-rays or thermal neutrons. Both measurements were expressed as percent of the control

Plant species	Age‡	Regression coefficients and their standard errors	
		X-ray treated	Thermal neutron treated
Barley (*Hordeum vulgare* L.)	11	2.20±0.54*	1.21±0.34
Maize (*Zea mays* L.)	12	2.06±0.47*	1.01±0.04†
Mustard (*Brassica juncea* Coss)	15	1.00±0.13†	0.61±0.11†
Safflower (*Carthamus tinctorius*)	19	1.33±0.15†	0.96±0.17†

* Significantly different from zero at the five percent level of probability.
† Significantly different from zero at the one percent level of probability.
‡ Days from planting to measurement of height.

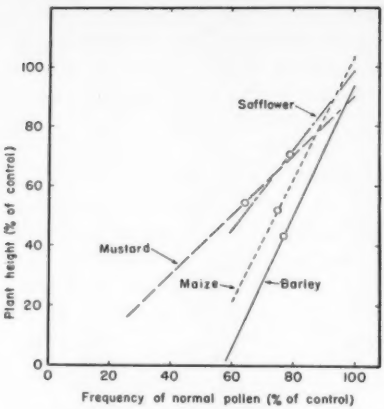

FIGURE 1.—Regression lines showing the relationship between plant height and percent normal pollen in four plant species whose dormant seeds were treated with varying doses of X-rays.

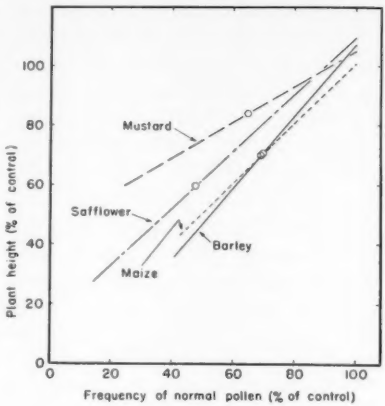

FIGURE 2.—Regression lines showing the relationship between plant height and percent normal pollen in four plant species whose dormant seeds were treated with varying doses of thermal neutrons.

(1957) suggested that the difference in linear energy transfer of the radiations was the direct cause of the differences in the biological effects of X-rays and fast or thermal neutrons. The variation between individuals within the X-rayed populations was shown to depend on a number of factors. Principal among them were moisture content of the embryo at the time of irradiation and environmental conditions during and after irradiation. Thus CALDECOTT (1958) has shown that it is possible to obtain a wide range in the frequency of mutations or meiotic chromosomal aberrations by controlling the water content of the dormant embryo before, during, and after irradiation. These later data appear to account, in a large measure, for the differences which were earlier attributed to an extrachromosomal or physiological effect of X-rays. Unpublished studies have shown that the X-ray induced injury to seeds of maize, oats, wheat, and tomatoes is subject to the same kinds of injury modification as barley. Thus it appears likely that the sensitivity of the seeds used in the present work may also have been modified by the environmental conditions preceding, during, or following X-ray treatment.

It is also apparent from the regression coefficients and lines that the four species differed somewhat in response to either kind of radiation, but that relative to one another they responded similarly to both kinds of radiation. For example, if the species are arranged in order of increasing regression coefficient, the same order is obtained for X-ray treatments as for thermal neutron treatments. However, the magnitude of the standard errors of the regression coefficients is such that one

must recognize the possibility that the responses of the four species within either kind of radiation were essentially alike.

The percentage of normal pollen shown in Tables 1, 2, 3, and 4 indicates that the dose of radiation required to produce a certain level of chromosomal abnormality differs widely for the four species. However, as may be seen from the regression lines in Figures 1 and 2, within either the X-ray or thermal neutron series, the four species are quite similar in the relationship between relative plant height and relative percentage of normal pollen. In other words, although the four species may differ widely in the sensitivity of the chromosomes to radiation, within each type of radiation the species appear remarkably alike with respect to influence on plant height per chromosomal abnormality.

SUMMARY

Seeds of barley, maize, mustard, and safflower were treated with various doses of X-rays and thermal neutrons, and observations of height, survival, and pollen abnormalities were made on the plants resulting from these seeds. In most cases a close relationship was found to exist between the three criteria of irradiation effect and dose of X-rays or thermal neutrons. However, it is necessary that care be used in selecting the age of plant at which height and survival data are taken.

The results suggest that although the chromosomes of the four species may differ widely in sensitivity to radiation damage, within each type of radiation the four species appear very similar with respect to influence on plant height per chromosomal abnormality.

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RELATION BETWEEN CHROMATID-TYPE AND CHROMOSOME-TYPE BREAKAGE-FUSION-BRIDGE CYCLES IN MAIZE ENDOSPERM

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BREAKAGE-FUSION-BRIDGE cycles have been rigorously proved to exist only in *Zea mays*, but there is every reason to believe that they occur widely. It is quite possible that they are universally produced by chromosomes broken in anaphase bridges. They may represent a major class of dominant lethals in genetic experiments and be responsible for the well-known observation that truly terminal deficiencies are not normally recovered in *Drosophila melanogaster*. A chromosome-type of breakage-fusion-bridge cycle has been described in the axolotl (DALTON and HALL 1950). In Agropyron, HAIR (1952) has described a chromosome-type breakage-fusion-bridge cycle in considerable detail. A similar chromosome-type breakage-fusion-bridge cycle has been found in Narcissus by DARLINGTON and WYLIE (1952). On other occasions, chromatin bridges persisting through several divisions have been plausibly attributed to breakage-fusion-bridge cycles of unidentified type. This is the case for the eggs of Habrobracon following irradiation of the sperm (WHITING 1945; ATWOOD, VON BORSTEL and WHITING 1956). KOLLER (1952) described a chemically induced tumor in the rat, which regularly had bridges, and ascribed by him to a chromosome-type breakage-fusion-bridge cycle. In the endosperm of Lilium BROCK (1954) has observed bridges almost certainly due to a breakage-fusion-bridge cycle. Finally, it should be noted that breakage-fusion-bridge cycles are probably an important mechanism in damage to growing tissues produced directly by radiation, as in the treatment of cancer.

Breakage-fusion-bridge cycles were first described by MCCLINTOCK (1938a). Chromatid bridges were generated at anaphase I of meiosis, from crossing over in an apocentric inversion, and the fate of the broken ends studied in gametophyte mitosis. In a series of papers by MCCLINTOCK since that date, many additional details were established; a brief general review, with diagrams of the two kinds of breakage-fusion-bridge cycle is given in MCCLINTOCK (1951). The first break initiating a breakage-fusion-bridge cycle can also be produced by a *Ds* element in the presence of *Ac*, or directly by radiation or some other mutagen. In the remainder of this paper, we shall simply use the word "cycle" as a convenient abbreviation for breakage-fusion-bridge cycle.

Two kinds of cycle are known, the chromatid type and the chromosome type respectively. The chromatid-type cycle results from a single break across one chromatid. This chromatid may consist of two strands which are broken at the

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same, or nearly the same level, the two free ends uniting. Alternatively, it might be considered that the chromatid is "single" at the time of breakage, but that when it doubles, the terminal element fails to duplicate, thus giving an equivalent sister union. The same result would be attained if the free ends are capable of rejoining immediately after duplication. Whatever its ultimate structure, the bridge that is produced at anaphase consists of one chromatid, which is either exactly or at least very nearly symmetrical about the preceding point of fusion.

The alternative chromosome-type cycle is initiated from a dicentric translocation. If the strands between centromeres are twisted through 180°, the anaphase figure will consist of two crossing chromatid bridges. With a 360° twist, or more half twists, interlocking chromatid loops result. These bridges break, and fusions occur between the ends of whole chromatids, thus re-establishing dicentric chromosomes in the two daughter nuclei.

It will be clear that each broken chromatid, in the chromosome-type cycle, could in principle undergo sister fusion, if its nature allows it to do so. If this were to happen, a chromosome-type cycle would be converted into two independent chromatid-type cycles. Conversely, two chromatid-type cycles might convert into a single chromosome-type cycle. The present paper is concerned with an experiment to test for the occurrence of this last event.

Information about cycles comes in part from direct cytological observations, in part from the phenotypes of the mosaics they yield. If the chromosome undergoing a cycle carries dominant markers, and the homologous normal chromosome carries the corresponding recessives, then a characteristic pattern is produced, as was first described by McCLINTOCK (1941b). While the geometry of the pattern varies widely, the *inclusion relation* of the loss areas, for a chromatid-type cycle, is a very definite one. It can be concisely stated in Set symbolism as: loss of distal marker $\supseteq$ loss of proximal marker (FABERGÉ 1956). This *inclusion relation* is simply a consequence of the breaking of a bridge which is symmetrical about its center with respect to the loci of the markers. Some infrequent accidents might upset this relation, such as breaking of a chromatid at several places, or perhaps the breaking of the sister strands at slightly different levels (McCLINTOCK 1945), but in practice such events are rare enough not to introduce any ambiguity. The inclusion relation does not necessarily hold for a chromosome-type cycle.

McCLINTOCK (1938a) showed that in the gametophyte, the chromatid-type cycle was the rule. In that paper, and in McCLINTOCK (1938b), it was shown that when two broken chromatids were introduced into the same nucleus, each of them continued, in the gametophyte, a separate chromatid-type cycle, and that fusions between broken chromatids to yield a chromosome-type cycle did not take place.

In contrast to the gametophyte, chromosome-type cycles were generated in the sporophyte when two broken ends were given to the zygote, one through the male, the other through the female gamete. This was first described by McCLINTOCK (1942), and later (McCLINTOCK 1943) an improved experimental arrangement yielded many more such unions. Earlier (McCLINTOCK 1938b) it had been shown

that the behavior of dicentric rings in the sporophyte required that the reunions of broken chromatids be of the chromosome, rather than of the chromatid or sister fusion type. This observation on ring chromosomes was later confirmed (McCLINTOCK 1941c).

If a single broken chromatid is introduced into the endosperm, it always undergoes a chromatid-type cycle. When introduced into a zygote, it will, ordinarily, stabilize; once stabilized, such a fresh chromosome end has the cytological properties of a natural chromosome end. However, this faculty of stabilizing a broken end may be under genotypic control, for in one experiment McCLINTOCK (1944a) observed among 188 cultures, four in which stabilization failed to occur, a chromatid-type cycle persisting throughout the development of the sporophyte. Chromosome-type cycles can also eventually stabilize in the late sporophyte development in maize (McCLINTOCK 1943). For this reason, it is to be expected that stable rod chromosomes should sometimes be recovered from ring chromosomes in maize, but so far this has not been observed (McCLINTOCK 1938b, 1941c).

The cytological possibilities of maize endosperm have intrinsic limitations, although bridges produced by cycles were demonstrated by CLARK and COPELAND (1940) and also by SCHWARTZ and MURRAY (in press). It is possible that bridges, particularly when short, tend to break early in anaphase and so escape detection.

McCLINTOCK (1941b) compared the endosperm mosaic patterns produced when one or when two chromosomes, each carrying the same dominant markers, have broken ends. Endosperm being triploid, such a comparison is conveniently made in a reciprocal cross, the broken chromosome being introduced either from the male or from the female parent. When two marked broken chromosomes were present, it appeared that the loss areas were larger than would be expected from a simple geometrical superposition of the patterns resulting from a single broken chromosome. Because of this apparent excess of loss area, McCLINTOCK suggested that the loss events in the case of two marked chromosomes might in fact not be independent. It was proposed that when two broken ends are introduced into the endosperm, they fuse and undergo a chromosome-type cycle, just as they had been proved to do in the zygote. Lack of independence in marker loss could then be brought about by a tendency for the chromatids of the double bridge to break at about the same level.

It has already been noted that the inclusion relation for marker losses does not necessarily apply to a chromosome-type cycle. The sister strand fusion of a chromatid-type cycle results in the bridge being symmetrical about its center with respect to the markers—with the possible rare exception of unequal breakage of the two strands. If a chromosome-type cycle is started by two broken chromosomes which are in every way identical, then the two bridges will also in the first instance be symmetrical, in the sense used above. The symmetry need not persist in later divisions, however, so that opportunities for departures from the inclusion relation will present themselves. McCLINTOCK's observation discussed in the preceding paragraph does not refer to such a departure, but only to a quantitative,

geometrical relationship. Looking for a departure from the inclusion relation, in the case of a chromosome cycle which was started as a symmetrical bridge would probably be very inefficient. A much more favorable situation would be created by a bridge that is asymmetrical from the outset.

In examining irradiated maize endosperm material, in which there were many mosaic kernels, it was noted that those attributable to cycles were, in the great majority of cases, of the regular types obeying the inclusion relation (FABERGÉ 1956). A plausible quantitiative consideration shows that most of them (about 89 percent) were probably taking place in the presence of other, unmarked broken chromosome arms. These would provide opportunities for fusions resulting in chromosome-type cycles that are from the beginning asymmetrical. While this radiation material provides no critical evidence, it suggests that chromosome-type cycles are not commonly formed in the endosperm. A preliminary description of the work presented here was given in FABERGÉ (1957).

MATERIALS AND METHODS

The aim of the experiment to be described here was to provide an opportunity for the formation of a chromosome-type cycle in the endosperm, in such a way that the event, if it occurred, could readily be recognized. The observations consisted in looking for a departure from the inclusion relation among marker losses in the mosaic. The positions of the breaks generated were such that any chromosome type cycle would from the beginning have bridges completely asymmetrical with respect to the markers. Such an experiment is easily made by means of MCCLINTOCK's (1949, 1951) displaced *Ds* stocks. Kernels of the constitution: *Ds C Sh Bz Wx/I Sh Bz Wx Ds/ I Sh Bz Wx Ds Ac/ac* were produced by making the cross: *I Sh Bz Wx Ds ac/ac* × *Ds C Sh Bz Wx/c sh bz wx Ac/ac*.

This experiment is similar in principle to the one described by MCCLINTOCK (1942, 1943) for the sporophyte, in that a broken chromosome is also introduced from the male and from the female gamete, and fusions between them looked for. An important difference is that in endosperm, because of the persistence of chromatid-type cycles, the opportunity for fusion is presented repeatedly at each cell division. In the sporophyte, on the other hand, this opportunity only occurs at zygote formation (or possibly in a few succeeding divisions). It will be noted that none of the crossover products can cause confusion. While there are four markers in the cross, in practice the relation between losses of *C* and losses of *Wx* is the critical one. Ideally, it would be possible to examine the same surface cells for both markers, but this is troublesome as the *C* anthocyanin pigment has to be bleached out before applying the iodine to detect *Wx*. It is more practical to make a very shallow cut, paring away the colored cells. This is legitimate, since the development of endosperm mosaics is chiefly radial, but the cut must be very shallow. To ensure the accurate superposition of the *C*-loss pattern with the *Wx*-loss pattern, holes about 0.08 mm in diameter were drilled in the vicinity of the areas being examined, and were filled with a fat soluble red pigment. These served as fiducial markers and can be seen on some of the illustrations. A vertical

illuminator was used for examining the kernels, and making Kodachrome records of typical cases. The illustrations (Figures 3–6) were made from the Kodachromes and are reproduced at a magnification of × 72.

RESULTS

About 1250 kernels of the critical constitution were obtained from a total of 8969 for the cross. Because their recognition depends on the presence of sufficiently large loss areas, the number is not exactly known. About 600 kernels had losses large enough to make detailed examination profitable and were actually used.

The two possible alternative kinds of chromosome behavior are illustrated in Figure 1. Starting with three homologous chromosomes broken at the *Ds* loci, the left side of the diagram shows each chromosome undergoing a separate independent chromatid-type cycle. The right side shows a paternal and a maternal chromosome fused to form a chromosome-type cycle; the remaining, third chromosome, is shown undergoing a chromatid-type cycle. It will be noted that at the stage when anaphase bridges are broken, the two sides of the diagram are identical, except that the broken chromosome ends may be nearer to each other in the case of the chromosome-type cycle. An opportunity for conversion of two chromatid-type cycles to a chromosome-type cycle is therefore presented at each cell division. For purposes of simplicity, the consequences of nondisjunction following bridge formation in endosperm (SCHWARTZ) will be disregarded at this stage and in the diagrams, but will be considered in the discussion.

Figure 2 shows one way in which a double bridge with asymmetrical strands in a chromosome-type cycle can break so as to give a loss of a proximal marker while preserving a distal one, or L*(C)* ⊆ L*(Wx)* on the mosaic pattern. A chromatid-type cycle in the male parent chromosome can only result in L *(C)* ⊇ L *(Wx)*, with the exception of the possible rare events mentioned earlier. The relative lengths of the segments of the short arm of chromosome 9 are represented about in proportion to their pachytene length, but the long arm is purely diagrammatic.

A kernel of the constitution shown is initially colorless because of the presence of *I*, one dose of which is enough to inhibit color. Colored areas on the kernel show that both female parent chromosomes have lost *I*, through breakage of *Ds* in the standard position. It is therefore known that cells in colored areas each carry two broken ends in chromosome arms that no longer carry any markers. These colored areas, which in typical cases occupy between a quarter and a tenth of the entire kernel surface, themselves show pronounced mottling and twin spotting. This is typical of the marker *C* when involved in a cycle, since the intensity of anthocyanin color increases with the dosage of *C*. Thus, it is known that the male parent chromosome carrying the markers *C Sh Bz Wx* is undergoing a cycle initiated by the break at the distal *Ds* locus. This cycle is occurring in the same cells in which two unmarked broken chromosome arms are present, and which are themselves presumably undergoing cycles whose presence cannot be demonstrated phenotypically. If the broken end of the male parent *C*-chromosome should fuse with one of the unmarked ends, a chromosome-type cycle would be initiated, as illu-

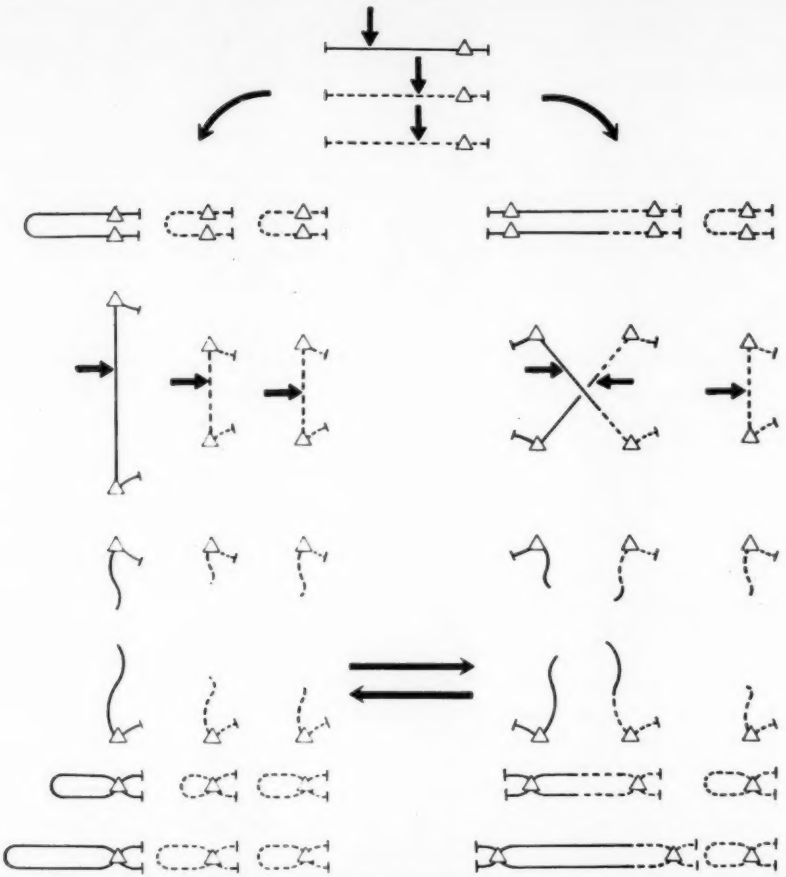


FIGURE 1.—Diagrammatic representation of the experiment. The male parent chromosome is shown in continuous line, the female parent chromosome in dotted line. On the left side, all three chromosomes are undergoing chromatid-type breakage-fusion-bridge cycles; on the right side, the paternal and a maternal chromosome have joined to form a chromosome-type breakage-fusion-bridge cycle. It will be noted that at the stage when bridges have broken, the two sides are structurally equivalent, so that a conversion as indicated by the large arrows is theoretically possible.

strated in the right side of Figure 1 and in Figure 2. Moreover, the opportunity for such a fusion is repeated at each cell division. In the case of a fusion occurring later, at some subsequent cell division, the relative lengths of some of the segments will differ from those shown in Figure 2, but the same principles will apply. Any bridge will be asymmetrical with respect to the markers.

The detectable phenotypic effect that may result from such a coalescence of

two chromatid-type cycles into a single chromosome-type cycle is the occurrence of a loss of *Wx* without previous loss of *C*. Many shallow cuts were made on colored areas and stained with iodine in the manner described, and no instances of such a loss of *Wx* underlying *C*-color were found. It is estimated that about 1000 cuts were made on approximately 600 kernels having sufficiently extensive colored areas. Since, at each cut, at least several hundred cells are examined, and losses of *Wx* as small as one cell are readily detectable, it can be estimated, as a rough order of magnitude, that if the event in question happens at all, it must be rarer than about one in 100,000 cell divisions. While this is a perfectly safe upper limit for the frequency, it seems impossible to make a closer estimate, because of the difficulty of assessing such subjective factors as the probability of not noticing a very small loss of *Wx* during the search. Moreover, this estimate of one in 100,000 cell divisions must certainly approach the frequency of various accidents in chromatid-type cycles, which were mentioned earlier, or the chance that a cut, while shallow, nevertheless went deep enough to uncover cells from a different cell lineage, or even mutation.

Associated with the colored regions in the kernels, were small colorless areas of obviously deficient cells. They were most often coupled with twin spotting, a darker colored area occurring by the side. The pattern of the cell outline shows that such areas were handicapped and grew more slowly than the surrounding tissue, as can be seen in Figures 3–6. These cells are homozygous deficient for all three ends of the short arm of the 9th chromosome, including the locus of *C*. Underlying such deficient, *colorless* areas, losses of *Wx* were often found. Several cases of this sort are illustrated in Figures 3–6. These small *Wx* losses were most often, of only three or four adjacent cells on the cross section, sometimes only one cell; a few as large as 10–12 cells were seen. They demonstrate that even quite small *Wx* losses in deficient tissue are readily detectable, and would undoubtedly have been seen under *C*-colored areas if they had occurred there. It would, more-

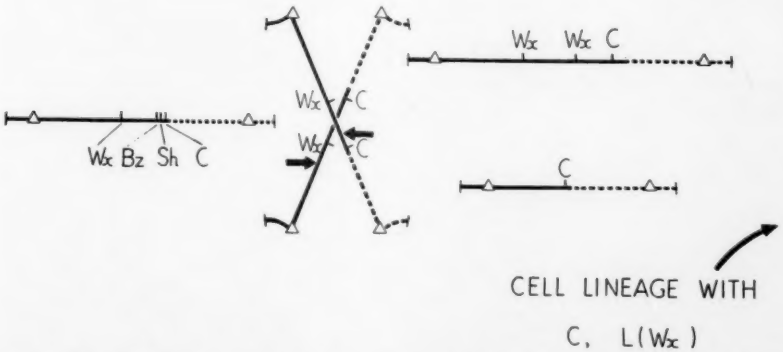
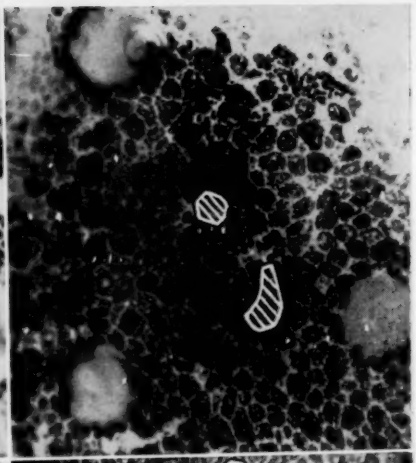


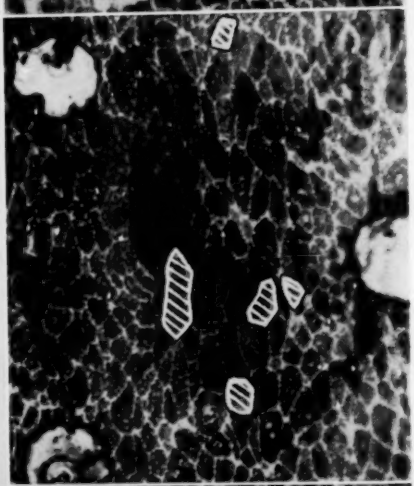
FIGURE 2.—Breakage of a double, chromosome-type bridge so as to yield the detectable event, loss of *Wx* without loss of *C*. The markers *C*, *Wx* are almost equidistant on the bridge.

3A

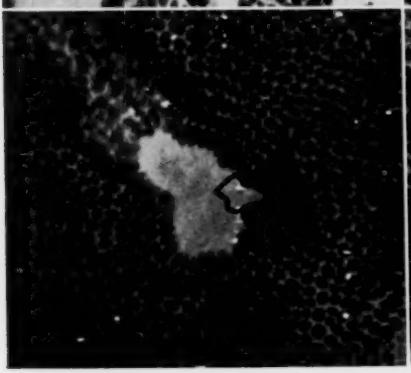


3B

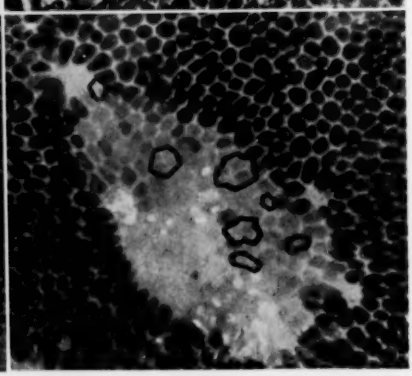
4A



4B



5



6

over, have been expected that any such losses of *Wx* underlying *C* must be less handicapped on the whole, and therefore larger, since they would be homozygous deficient for a smaller part of the chromosome arm. This is an attempt to estimate the maximum frequency with which the phenotypic event might occur and still pass unrecognized. To pass from this to the actual frequency of formation of a chromosome-type cycle requires division by the probability that a double bridge breaks in such a manner as to produce the event $L(C) \subseteq L(Wx)$ on the mosaic. There is little sound basis for making such an estimate with any accuracy. If we assume that the probability of breakage is uniform throughout the entire length of each strand of the bridge, and consider the initial bridge, as illustrated in Figure 2, the probability of the critical event is 17.6 percent. This has to be multiplied by probability of a half-twist between the centromeres, though interlocking strands with an odd number of half-twists can also generate the critical event; in that last case the assumption of uniform distribution of breaks along the strand is perhaps rather less likely to hold. For bridges in later cell lineages, when the lengths of some of the segments would be different from those in the intial bridge, the probabilities will obviously be different. They may be greater or smaller. Mosaics clearly show that a bridge does not break in any regular position, but it does not follow that there is exactly uniform probability of breakage along the length. Departures from uniformity may again either increase or decrease the probability of occurrence of the critical event. The entire situation seems too complex to permit a realistic calculation on that basis.

There is, however, a much simpler and more satisfactory way of assessing the approximate probability of detection of a chromosome-type cycle in the experiment. It is based on the fact that the two markers *C* and *Wx* would lie nearly symmetrically in the initial dicentric bridge, and thus should be equally exposed to the probability of loss. It will be seen from Figure 2, that *C* and *Wx* are at about the same distance from the center of the bridge and from the centromeres. In later cell divisions this will change, but changes favoring loss of *C* will be equal to changes favoring loss of *Wx*. If the markers were exactly equidistant, then the probability of $L(C) \supseteq L(Wx)$ would equal $L(C) \subseteq L(Wx)$. Since $L(C) \supseteq L(Wx)$ is observable several times on each kernel, but the other type of event is not, it seems quite safe to conclude that $L(C) \subseteq L(Wx)$, if it ever occurs, must be rarer than one in several thousand.

It is concluded that when several broken chromosomes are present simultane-

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FIGURES 3–6.—Magnification ×72. 3A, 4A regions of aleurone surface with losses of *C*, underlying losses of *Wx* drawn in thick outline. 3B, 4B corresponding areas after paring off surface cells and staining with iodine; losses of *Wx* outlined. 5, 6 aleurone surface with losses of *C*, losses of *Wx* outlined. Large spots near margins are markers to ensure coincidence of photographs of surface with those of cut areas.

ously in maize endosperm tissue, each arm undergoes an independent chromatid-type cycle. A chromosome-type cycle is not formed between two such broken ends at a detectable frequency, in the way it is formed in the maize sporophyte.

DISCUSSION

It cannot be claimed that the behavior of broken chromosomes is fully understood even in maize. The general picture presented by a review of the literature is fairly complex and not entirely free of contradiction. It seems worth-while, nevertheless, to formulate some very tentative generalizations, in the hope that they may lead to further experiments.

An obvious question is why, under some conditions, chromatid-type cycles are formed, while under other conditions, a chromosome-type cycle is the rule. McCLINTOCK has touched on this question in several of the papers that have been quoted. A possible speculation is that this is related to effective singleness or effective doubleness of a chromatid. By *effective*, in this context, is to be understood *effective with respect to the possibility of sister strand fusion*. It does not necessarily mean that a chromatid, in some tissues, actually has only one strand while in others it has two or four. It might mean, for instance, that in one case the sister strands are prevented from fusing by a protective matrix, or by a special state of spiralization while in another case they are free to fuse.

It is clear that when a double, chromosome-type bridge has broken, the ends of sister strands are always much nearer each other than are the ends of whole chromosomes. Many different results in radiation work strongly indicate that initial separation of free ends is of critical importance in rejoining, and it may be remarked that in a crude kinetic model which considers free ends as independent particles, the time to contact will be some function of $(\text{distance})^{-3}$. While such models are unrealistic in disregarding steric considerations, they serve to demonstrate the great importance of initial separation. Thus, if sister fusion can occur at all easily, it ought to take precedence over chromosome fusion in cycles, and a chromosome cycle should convert to a chromatid cycle.

This may be the reason why a chromosome cycle is not formed in the gametophyte, a tissue in which McCLINTOCK (1938a) had reported suggestions of actual visible doubleness in chromatids. Such visible doubleness in anaphase chromatids has been seen in other materials by many workers, among whom might be mentioned NEBEL (1941) and MANTON (1945). It does not seem impossible that some of the double stranded bridges reported by SCHWARTZ and MURRAY (in press) are of the same nature. It is thus conceivable that the same considerations apply to early endosperm divisions as to the gametophyte. However, following nondisjunction, a single chromatid cycle can convert to a chromosome cycle in endosperm, at least in the later divisions, showing that a chromosome cycle can under these conditions compete against two chromatid cycles (SCHWARTZ and MURRAY in press). A chromosome-type cycle, even when initially symmetrical with respect to markers should, after some divisions, sometimes produce departures from the inclusion relation on the mosaic, since it seems unlikely that the two strands

of the bridge will always break at exactly the same level. Such departures being very rarely observable, it seems likely that most of the visible pattern is determined before nondisjunction occurs. This conclusion is reinforced by the consideration that nondisjunction would result in an area of loss of all markers at least equal to the area showing other losses, which is certainly not the case in the early, large scale, mosaic patterns. Many anaphases in endosperm show no visible bridges, but it is impossible to decide to what extent this may be due to failure of sister fusion or to early breaking of the bridge.

There are indirect indications from radiation data (FABERGÉ 1956) of a strong bias against interchromosomal exchanges in maize endosperm. This factor may be mainly responsible for the results presented here.

It would be out of place to discuss at length the relation of cycles to the behavior of radiation-induced chromosome breaks. As far as singleness or doubleness of strands is concerned, there is substantially nothing to add to MCCLINTOCK's (1938a) discussion. In the male gametophyte of maize, sister fusion always (or almost always) follows breakage at anaphase. In the corresponding stage of Tradescantia and some other plants, if breaks are induced at the "chromosome break" phase, that is, between meiotic telophase and about 30 hours preceding microspore metaphase, sister fusions are rare or absent. The chromosome behaves with respect to X-rays as though mainly single. Whether the rarity of sister fusions at this stage is due to a true stabilization of the breaks, or alternatively to sister fusion of *subchromatids* has never been established. If the last possibility is correct, bridges ought to appear in the pollen tube division following irradiation immediately after meiosis, but attempts to demonstrate them have failed because of technical difficulties (BISHOP 1950).

It is of course entirely possible that chromosome organization is different in maize and in the other plants mentioned. We would be reluctant to accept such a view without examining some other alternatives. In particular, it does not seem impossible to imagine a plausible model of chromatid structure which will be capable of passing from a double to a single phase. For instance, if a matrix is much less extensible than the strands embedded in it, these strands might be freed and approximated when the whole structure is stretched in a bridge, but effectively kept apart when not under tension.

A closely related question is that of the nature of true stabilization of free ends. It might be supposed that the necessary condition is simply a sufficiently long sojourn of a free end in the nucleus, in the unsaturated state. Because of the great difference in distance, it seems likely that sister fusion can follow breakage much more rapidly than does a chromatid reunion. It might thus be expected that in any cell division broken ends remain unsaturated much longer under a chromosome cycle regime than under a chromatid-type cycle regime. Thus, chromosome cycle conditions might be generally associated with the possibility of stabilization. The chromosome-type cycle of HAIR (1952) sometimes underwent stabilization in somatic tissues, just as in maize. It seems possible that the cycle in Narcissus (DARLINGTON and WYLIE 1952) also did so.

SUMMARY

An experiment is described whose purpose was to ascertain whether, in maize endosperm, separately broken chromosomes will fuse to generate a composite chromosome-type breakage-fusion-bridge cycle. Markers in the short arm of chromosome 9 were used, and breaks were induced by the *Ds-Ac* system. The markers and positions of breaks were such that if reunion between two broken chromosomes occurred, the event would be phenotypically recognizable on the endosperm mosaic. It was found that each broken chromosome end undergoes a breakage-fusion-bridge cycle (presumably initially of chromatid type) on its own and that fusion of different broken chromosome ends to give a composite chromosome-type breakage-fusion-bridge cycle does not occur in endosperm, at a detectable frequency. In this respect, endosperm differs from the sporophyte but is similar to the gametophyte.

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A STATISTICAL STUDY OF THE INHERITANCE OF STONEVILLE 20 RESISTANCE TO THE BACTERIAL BLIGHT DISEASE OF COTTON IN THE PRESENCE OF XANTHOMONAS MALVACEARUM RACES 1 AND 2[1]

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UTILIZING inherently resistant plants is the only known method for adequately controlling the bacterial blight disease of cotton, which is caused by *Xanthomonas malvacearum* (E. F. Sm.) Dowson. For this reason the development of resistant varieties has received considerable attention during the past decade.

In 1939 SIMPSON and WEINDLING (1946) found a selection from Stoneville 2A which was resistant to bacterial blight. The selection was designated Stoneville 20. This strain has been the principal source of resistance used in a number of breeding programs in the United States. SIMPSON[3] reported that Stoneville 20 resistance was inherited as a simple recessive character with susceptibility being dominant and that a favorable alignment of modifying or minor genes was necessary for the full expression of resistance. BLANK (1949) reported studies which supported SIMPSON's hypothesis. BIRD (1950) conducted experiments involving Stoneville 20 resistance in a segregating population following the second backcross to Stoneville 2B. It was shown that heterozygous plants could be distinguished from homozygous ones and that if dominance was present it was in the direction of resistance. The gene was designated B' until its relationship with KNIGHT's and CLOUSTON's (1939) factors B_1 and B_2 and KNIGHT's (1944, 1948) factors B_3 and B_4 could be ascertained. After studying segregating populations from third backcross generations in Deltapine and Acala material, BIRD and BLANK (1951) concluded that Stoneville 20 resistance was recessive but showed that heterozygous plants tend to be intermediate between the homozygous ones when minor genes were present. KNIGHT (1953), after studying Stoneville 20 resistance in a Sakel background, concluded that it was inherited as a simple

[1] Cooperative investigations between Texas Agricultural Experiment Station and C.R.D., A.R.S., United States Department of Agriculture. The data discussed in this paper formed a portion of a dissertation which was presented to the Graduate School of the A. & M. College of Texas as partial fulfillment of the requirements for the degree of Doctor of Philosophy, May, 1955.

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[3] In correspondence with L. M. BLANK, College Station, Texas, 1946.

dominant character, and he designated the gene as B_7. He emphasized the importance of minor genes in its expression.

The information available on the inheritance of resistance in Stoneville 20 makes it possible to study and apply statistical methods for partitioning the phenotypic variance into its fixable genetic, unfixable genetic and nonheritable components. It also offers opportunity for determining whether or not inheritance studies of resistance can be resolved statistically with a high degree of accuracy where disease grades are used for measuring phenotype. These theoretical studies could provide additional information for planning a more efficient breeding program for developing resistant varieties with Stoneville 20 as the source of resistance.

MATERIALS AND METHODS

Four strains of *Gossypium hirsutum* L. were used as parents for the genetic studies. They were Stoneville 20 (P_1), Deltapine (P_2), Stoneville 2B (P_3), and Acala (P_4). These were selected because of their differential response to infection by *X. malvacearum*. Stoneville 20 is resistant, Deltapine has high tolerance, Stoneville 2B has low tolerance and Acala is susceptible. The differences in tolerance of the susceptible strains is attributed to different levels of minor genes.

The four parents were planted in the field in 1950 and the crosses $P_1 \times P_2$, $P_1 \times P_3$, and $P_1 \times P_4$ were made. Eight F_1 plants from each cross were grown in the greenhouse during the winter of 1950–1951. In 1951 the four parents, the three F_1's and F_2's were planted. Each F_1 was backcrossed to each of its parents. The F_2 plants were self-pollinated. Fifty F_2 plants were selected from each cross, on the basis of sufficient self-pollinated seed for planting, for production of F_3 families in 1952. A bulk F_3 was made by combining seven seeds from each of the 50 F_2 plants per cross. The seeds were mixed and then divided into the necessary number of lots for planting.

In 1952, three entries of each parent, three of each F_1, six of each F_2, six of each bulk F_3, three of each of the six backcrosses and 50 F_3 families for each cross were arranged in a 15×15 simple lattice design with two replications of single row plots 25 feet long. A perfect stand would have given 20 plants per plot. The 1951 and 1952 plantings were made at the same location on the Main Station Farm, near College Station, Texas.

The 1951 and 1952 field plantings were inoculated by using the method reported by BIRD and BLANK (1951). Three to four weeks after inoculation each plant was assigned a disease grade by using the grading system shown in Figure 1. The 1951 inoculum was prepared by compositing growth of three *X. malvacearum* race 1 isolates which were designated T_1, T_4 and T_{12}. The 1952 inoculum was prepared from growth of two isolates which were designated T_{12} (race 1) and G_4 (race 2).

The components of variation in backcrosses and in F_2 and its derivatives which were used for partitioning the 1952 variances are given in Table 1.

It was learned in 1953 from work done by HUNTER and BLANK (1954) that the *X. malvacearum* isolate T_{12} was race 1 and G_4 was race 2. For this reason in 1954

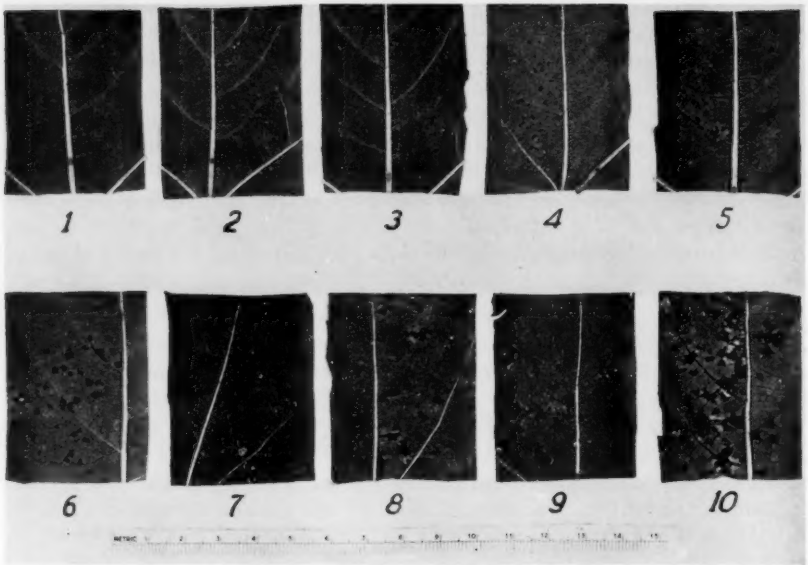

FIGURE 1.—Disease grading system. Grades: 1, no infection; 2, lesions about 0.5 mm in size, round and raised, brown, never angular, wet or coalesced; 3, intermediate to grades 2 and 4; 4, lesions about 1 to 1.5 mm in size, round to slightly angular and sunken, never coalesced; 5, intermediate between 4 and 6; 6, lesions 2 to 3 mm in size, angular, and sunken, some coalescing; 7, lesions 2 to 5 mm in size, angular with coalescing, exudate present; 8, intermediate to 7 and 9; 9, lesions 3 to 7 mm in size, frequent coalescing, exudate present; 10, lesions 3 to 10 mm in size, extensive coalescing, exudate present.

a population of 24 Stoneville 20 plants and an F_2 population of 54 plants from the cross Stoneville 20 × Stoneville 2B were studied to ascertain the response of the populations to the two races. This was done by separately inoculating each plant with each isolate and then grading the resulting infection.

RESULTS

An analysis of variance of the plot means for the 1952 15 × 15 simple lattice test was made. The mean squares for replications and blocks was highly significant. For this reason replication and block differences were removed from all variances before using them for further calculations. The mean square for strains was also highly significant.

The statistics for the generations following the crosses $P_1 \times P_2$, $P_1 \times P_3$, and $P_1 \times P_4$ which were grown in 1951 and 1952 are given in Table 2. The mean of the P_1, resistant parent, was larger (more susceptible) in 1952 than it was in 1951. The means for the P_2, P_3, and P_4, susceptible parents, and the F_1's and F_2's were smaller (more tolerant) in 1952 than they were in 1951. The standard deviations

for both years with the exception of the F_2's, which were smaller in 1952, were about the same.

The F_2 frequency distributions for the three crosses for 1951 and 1952 are shown in Figure 2. The ranges for the F_2's were smaller in 1952 than they were in 1951. The F_2 frequency distribution in 1951 for the cross $P_1 \times P_2$ was continu-

TABLE 1

Components of variation in backcrosses and in F_2 and its derivatives which were used for partitioning the 1952 variances

Variance*	Source of Variation	Components
V_{F_2}	F_2 variance	$1/2D+1/4H+E_1$
$V_{\bar{F}_3}$	Variance of means of F_3 progenies	$1/2D+1/16H+E_2$
W_{F_2/F_3}	Covariance of F_3 mean and F_2 parental measurement	$1/2D+1/8H$
$\bar{V}_{F_3}$	Mean variance of F_3 progenies	$1/4D+1/8H+E_1$
V_{BF_3}	Variance of bulk F_3 progenies	$3/4D+3/16H+E_1$†
$V_{B_1}+V_{B_2}$	Sum of variances of B_1 and B_2	$1/2D+1/2H+2E_1$
E_1	Nonheritable variance of plants within a plot	E_1
E_2	Nonheritable variance of plot means round the block mean	E_2

* With the exception of V_{BF_3} representing variance of bulk F_3 the symbols used are the same as those given by MATHER (1949).
† Suggested by the junior author.

TABLE 2

Statistics for each generation of the three crosses which were grown in 1951 and 1952

	$P_1 \times P_2$			$P_1 \times P_3$			$P_1 \times P_4$		
Generation	N	Mean*	S.D.	N	Mean†	S.D.	N	Mean‡	S.D.
				1951					
P_1	94	1.5000	1.1330	94	1.5000	1.1330	94	1.5000	1.1330
F_1	106	4.9200	1.1070	112	4.5300	0.8270	96	7.1400	1.0720
F_2	86	4.6100	1.4800	83	5.1700	1.9110	95	6.6500	1.8890
P_2, P_3 or P_4	115	6.1800	0.7820	90	6.9800	0.7490	82	8.3700	0.9880
				1952					
P_1	119	2.9244	1.0148	119	2.9244	1.0148	119	2.9244	1.0148
B_1	84	3.0714	1.2007	92	2.7174	1.1226	71	4.3380	1.3425
F_1	116	2.9310	1.0037	119	3.2269	0.8133	105	5.1429	0.9494
F_2	229	3.2358	1.2443	239	3.4770	1.2008	240	4.3167	1.3592
F_3 bulk	217	3.7097	1.1794	232	3.5216	1.1235	224	4.5982	1.3102
F_3 families	1710	3.5281	1.0481	1587	3.4297	1.0049	1462	4.9829	1.0044
B_2	109	3.6514	0.9569	98	3.5612	0.9794	101	5.7129	0.8116
P_2, P_3 or P_4	120	4.6417	0.6943	119	3.6218	0.7679	119	6.4286	0.7564

* Mid-parent mean for 1951: 3.8400; for 1952: 3.7831.
† Mid-parent mean for 1951: 4.2400; for 1952: 3.2731.
‡ Mid-parent mean for 1951: 4.9350; for 1952: 4.6765.

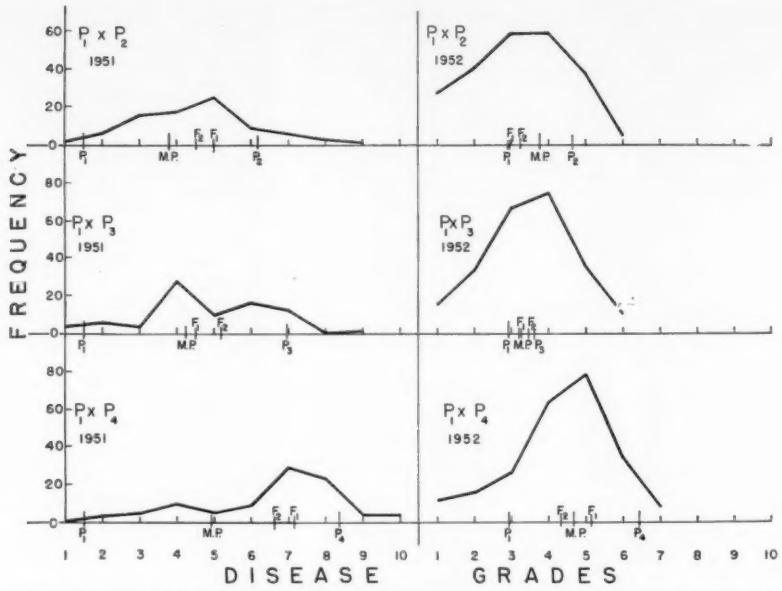

FIGURE 2.—The F_2 frequency distributions for 1951 and 1952 for the crosses $P_1 \times P_2$, $P_1 \times P_3$ and $P_1 \times P_4$. M. P. represents mid-parent value.

ous, for the cross $P_1 \times P_3$ it was trimodal while the F_2 from the cross $P_1 \times P_4$ was bimodal. The 1952 frequency distributions for the three crosses were continuous.

The F_1 means, in 1951, for the crosses $P_1 \times P_2$ and $P_1 \times P_4$ were intermediate to the values for the mid-parent and the susceptible parent, while for the cross $P_1 \times P_3$ the F_1 mean and the mid-parent values were about the same. In 1952 the F_1 mean of the cross $P_1 \times P_2$ was the same as the mean of the P_1. The F_1 mean for the cross $P_1 \times P_3$ was about the same as the mid-parent value, while the F_1 mean for the cross $P_1 \times P_4$ was about intermediate to the mid-parent value and the P_4.

The frequency distributions of disease grades for the Stoneville 20 population which was studied in 1954 by separate inoculations with the *X. malvacearum* isolates T_{12} and G_4 are given in Table 3. Similar results for the F_2 population from the cross Stoneville 20 × Stoneville 2B are given in Table 4. Stoneville 20 was more resistant in the presence of isolate T_{12} than it was in the presence of isolate G_4, as shown by the higher mean disease grade for the latter isolate. When the highest disease grade of either isolate was used to represent a plant, the mean disease grade was the same as that for isolate G_4. The range for isolate T_{12} was from grade 1 to grade 4 while the ranges for G_4 and the combined results were from grade 2 to grade 6. The standard deviations for T_{12}, G_4 and the combined results were the same. For the F_2 population the mean disease grade for G_4 and

the combined results tend to be higher than the mean for T_{12}. The range for T_{12} is from grade 1 to grade 8, for G_4 it is from grade 1 to grade 7, and for the combined it is from grade 2 to grade 8. The distribution for isolate T_{12} is tri-modal while for G_4 and the combined results it is continuous. The standard deviation for T_{12} is larger than that of G_4 and the combined results which are the same. The F_2 results suggest that in the presence of isolate T_{12} (race 1) Stoneville 20 resistance is a simple mendelian character with no dominance. However, in the presence of isolate G_4 (race 2) or both T_{12} and G_4 resistance appears to be controlled by several factors.

Tests were made with the 1952 data to determine the adequacy of the scale for measuring bacterial blight resistance. Results of the tests for the three crosses in regard to genetic interaction are given in Table 5. For the crosses $P_1 \times P_2$ and $P_1 \times P_3$ the A, B and C values are not significant. For the cross $P_1 \times P_4$ the A and B values are not significant, but the C value is. Results of the tests with the 1952 data for the three crosses in regard to independence of nonheritable variation from the genotype are given in Table 6. For the crosses $P_1 \times P_2$ and $P_1 \times P_4$ the variances for the P_1 and F_1 are not different from the variances of the susceptible parents. For the cross $P_1 \times P_3$ the variances of the F_1 and P_3 are not different, but both are different from the variance of the P_1. A similar study of the 1951 data shows that the crosses $P_1 \times P_2$ and $P_1 \times P_3$ behaved the same as they did in 1952. However, in 1951 for the cross P_1 and P_4 the variances for the P_1, F_1, and P_4 were the same.

For partitioning the variance into the four components fixable genetic (D),

TABLE 3

Frequency distributions of the disease grades for the Stoneville 20 population which was studied in 1954 by separate inoculations with two isolates

Isolates	Disease grades										Statistic		
	1	2	3	4	5	6	7	8	9	10	N	Mean	S.D.
T_{12}	14	4	3	3	.	.	.	.	.	.	24	1.79	1.10
G_4	.	3	3	8	7	3	.	.	.	.	24	4.17	1.20
Combined*	.	2	4	8	7	3	.	.	.	.	24	4.21	1.14

* Where the highest disease grade of either isolate is used to represent a plant.

TABLE 4

Frequency distributions of disease grades for the F_2 population from the cross Stoneville 20 × Stoneville 2B which was studied in 1954 by separate inoculations with two isolates

Isolates	Disease grades										Statistic		
	1	2	3	4	5	6	7	8	9	10	N	Mean	S.D.
T_{12}	7	5	4	15	8	9	4	2	.	.	54	4.20	1.93
G_4	1	3	7	19	13	8	3	.	.	.	54	4.41	1.31
Combined*	.	2	3	14	14	13	6	2	.	.	54	5.09	1.32

* Where the highest disease grade of either isolate is used to represent a plant.

TABLE 5

Test for adequacy of scale used in rating bacterial blight resistance in the generations following the three crosses $P_1 \times P_2$, $P_1 \times P_3$ and $P_1 \times P_4$ in regard to genetic interaction

Generations	$P_1 \times P_2$		$P_1 \times P_3$		$P_1 \times P_4$	
	Mean	S.E.	Mean	S.E.	Mean	S.E.
P_1*	2.9244	.2961	2.9244	.2961	2.9244	.2961
B_1	3.0714	.1791	2.7174	.1540	4.3380	.0853
F_1	2.9310	.2743	3.2269	.2686	5.1429	.0457
F_2	3.2358	.1253	3.4770	.1159	4.3167	.1178
B_2	3.6514	.3740	3.5612	.4039	5.7129	.3182
$P_{2'3'4}$†	4.6417	.1917	3.6218	.1978	6.4286	.1684
A	0.2874	.5394	—0.7165	.5047	0.6087	.3450
B	—0.2699	.8195	0.2737	.8739	—0.1457	.6601
C	—0.4849	.8225	0.9080	.7937	—2.3720‡	.5890

* Bacterial blight resistant Stoneville 20.
† Bacterial blight susceptible parents.
‡ Significant at the one percent level.

TABLE 6

Test for adequacy of scale used in rating bacterial blight resistance in generations following the three crosses $P_1 \times P_2$, $P_1 \times P_3$ and $P_1 \times P_4$ in regard to independence of the magnitude of nonheritable variation from the genotype

Source of variation	Degrees of freedom	Variance	F	
			Comparison one*	Comparison two†
		$P_1 \times P_2$		
P_1	118	1.0298	2.14‡	1.02
F_1	115	1.0074	2.09‡	
P_2	119	.4821		
		$P_1 \times P_3$		
P_1	118	1.0298	1.75‡	1.56‡
F_1	118	.6615	1.12	
P_3	118	.5897		
		$P_1 \times P_4$		
P_1	118	1.0298	1.80‡	1.14
F_1	104	.9014	1.58‡	
P_4	118	.5721		

* P_2, P_3 and P_4 compared with their respective F_1's and the P_1.
† F_1's compared with P_1.
‡ Exceeds the one percent level of significance.

unfixable genetic (H), nonheritable within plot (E_1) and nonheritable between plot means (E_2), the mean variances for the two replications were used. The series of eight statistics were set equal to the obtained mean variances for the two replications. The series of basic equations were then solved for the D, H, E_1, and E_2 estimates as outlined by MATHER (1949). These values where all available variances were used for the calculation are given in the top group in Table 7. The D values for each of the three crosses are significant. The H values are all positive

TABLE 7

D, H, E_1 and E_2 values for the three crosses $P_1 \times P_2$, $P_1 \times P_3$ and $P_1 \times P_4$‡

Components	$P_1 \times P_2$		$P_1 \times P_3$		$P_1 \times P_4$	
	Component	S.E.	Component	S.E.	Component	S.E.
	Where all variances are included					
D	1.4508†	0.4391	1.4743*	0.5067	1.8976*	0.5191
H	1.4058	1.3532	1.6622	1.5615	0.4284	1.6022
E_1	0.4690	0.2399	0.3256	0.2768	0.5338	0.2840
E_2	0.4018	0.2321	0.5067	0.2678	0.2517	0.2748
	Where $\overline{V}_{F_3}$, V_{BF_3} and $V_{B_1}+V_{B_2}$ are excluded					
D	2.5689*	1.0039	2.9121*	1.1104	2.2026*	0.7460
H	—1.1043	3.2123	—1.7409	3.5530	0.4845	2.3872
E_1	0.7000	0.3011	0.6019	0.3331	0.7397*	0.2238
F_2	0.2007	0.2749	0.2536	0.3041	0.1737	0.2043

* Significant at the five percent level.
† Significant at the one percent level.
‡ For detailed data and matrix of multipliers used see appendix tables in a dissertation entitled, "The bacterial blight disease of cotton. III A statistical study of the inheritance of Stoneville 20 resistance. IV The physiological nature of Stoneville 20 resistance." The Library, A. & M. College of Texas, College Station, Texas, May 1955.

but not significant. Likewise the E values are not significant, although the E_1 values approach significance.

In order to conduct linkage tests as described by MATHER (1949), D, H, and E values were also calculated where V_{F_3}, V_{BF_3}, and $V_{B_1}+V_{B_2}$ were excluded from the calculations. The D, H, and E values are given as the bottom group in Table 7. The D values are significant. The H values for the crosses $P_1 \times P_4$ is positive. However, none of them is significant. The E_1 value for the cross $P_1 \times P_4$ is significant while the other E values are not.

Linkage tests for the three crosses where the V_{F_3}, V_{BF_3}, and $V_{B_1}+V_{B_2}$ were excluded were made. The linkage mean squares for the crosses $P_1 \times P_2$ and $P_1 \times P_3$ were significant. The residual interaction mean squares for the same crosses were highly significant. Neither the linkage nor residual interaction mean squares for the cross $P_1 \times P_4$ were significant. This is because the replication mean square was so high. Its size could be attributed to an exceptionally low variance for one plot of the B_2 in replication two. If it had not been for this chance variation which caused a large error mean square, linkage and interaction for the cross $P_1 \times P_4$ would also have been significant. The results suggest that the genes for bacterial blight resistance which were involved in the three crosses are linked. However, the fact that the residual interaction was highly significant casts doubt on this interpretation. According to MATHER (1949) a significant residual interaction also suggests that the scale may not be adequate.

The theoretical means for determining whether gene action is additive or geometric were calculated by formulas given by POWERS and LYON (1941). The observed and theoretical means based on arithmetic and geometric gene action in F_1, F_2 and reciprocal backcross populations for the crosses $P_1 \times P_2$, $P_1 \times P_3$, and $P_1 \times P_4$ are given in Table 8. The theoretical arithmetic and geometric means are essentially the same and differ very little from the observed means.

Heritability of differences among F_2 plants was estimated by three different methods. These estimates are presented in Table 9. Frequency distributions of the means of F_3 families in 1952 for the crosses $P_1 \times P_2$, $P_1 \times P_3$ and $P_1 \times P_4$ are shown in Figure 3. The correlation coefficients between the phenotype of the F_2 plants and the F_3 family means for the crosses $P_1 \times P_2$, $P_1 \times P_3$ and $P_1 \times P_4$ were

TABLE 8

Observed and theoretical means based on arithmetic and geometric gene action for the three crosses $P_1 \times P_2$, $P_1 \times P_3$ and $P_1 \times P_4$

Cross and generation	Observed	Theoretical	
		Arithmetic	Geometric
$P_1 \times P_2$			
F_1	2.931±0.093	3.778	3.685
F_2	3.236±0.082	3.357	3.286
B_1	3.071±0.131	2.928	2.929
B_2	3.651±0.091	3.787	3.789
$P_1 \times P_3$			
F_1	3.227±0.075	3.273	3.254
F_2	3.477±0.078	3.250	3.241
B_1	2.717±0.117	3.076	3.074
B_2	3.561±0.099	3.425	3.419
$P_1 \times P_4$			
F_1	5.143±0.093	4.676	4.335
F_2	4.317±0.088	4.910	4.722
B_1	4.338±0.159	4.034	3.878
B_2	5.713±0.081	5.786	5.750

TABLE 9

Heritability of differences among F_2 plants for the three crosses where different methods of calculation were used

Formula	Year of data	Cross		
		$P_1 \times P_2$	$P_1 \times P_3$	$P_1 \times P_4$
Regression*	1951–52	.48	.43	.31
$\frac{1/2D}{1/2D+1/4H+E_1}$†	1952	.49	.48	.85
	1952‡	.47	.51	.51
	1952§	.47	.50	.60
$\frac{V_{F_2}-V_{F_1}}{V_{F_2}}$‖	1951	.44	.81	.68
	1952	.35	.54	.51

* After LUSH (1948), regression of mean of F_3 family on its F_2 plant measurement.

† After WARNER (1952), where 1/2D is calculated as $2V_{F_2} - V_{B_1} + V_{B_2}$.

‡ Where the D values (all variances included) given in Table 7 are used and where the obtained V_{F_2} was used as the denominator.

§ Where the D values (all variances included) given in Table 7 are used and where the expected V_{F_2} was used as the denominator.

‖ After BURTON (1951).

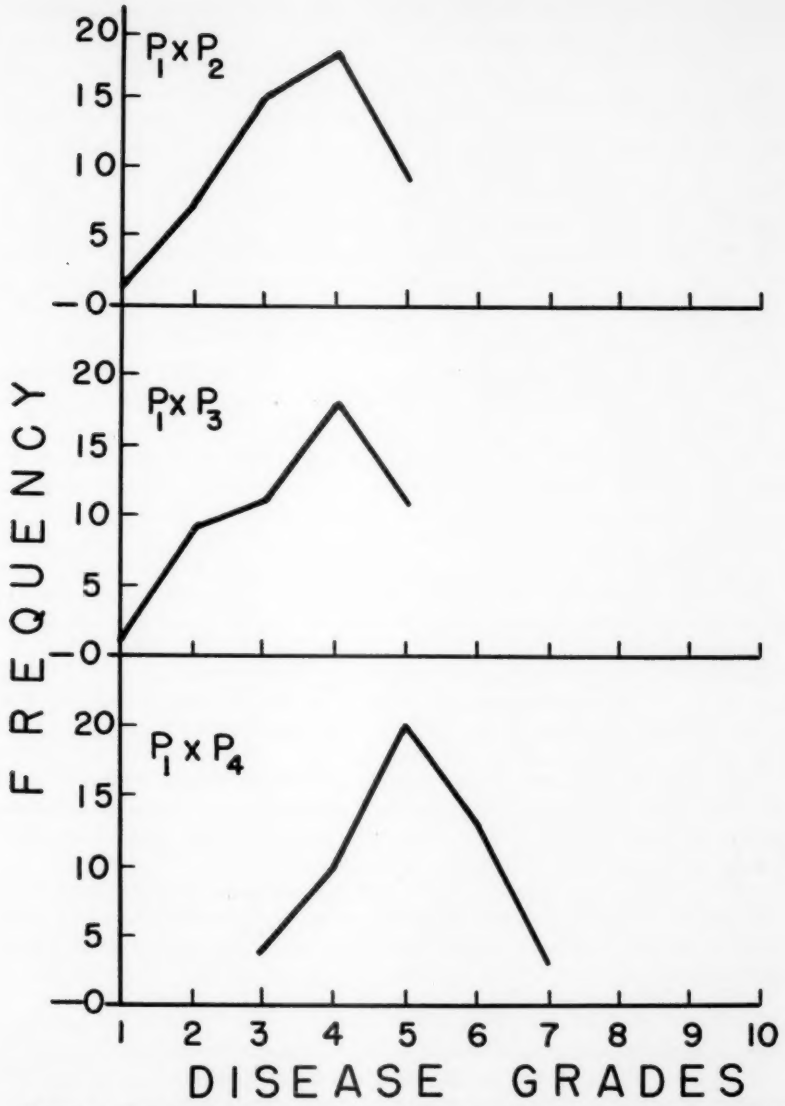

FIGURE 3.—The frequency distributions of F_2 family means which were grown in 1952 for the crosses $P_1 \times P_2$, $P_1 \times P_3$ and $P_1 \times P_4$.

respectively 0.84, 0.78 and 0.65. By comparing the 1951 frequency distributions of F_2 plants shown in Figure 2 with the 1952 frequency distributions of F_3 means one would expect for the regression method of calculating heritability, the value for the cross $P_1 \times P_2$ to be higher than the $P_1 \times P_3$ value which in turn should be higher than the P_1 and P_4 value. Therefore, the results in Table 9 conform to the expected. In observing the frequency distributions for the 1952 F_2's and the F_3 means, one would expect the heritability values for the three crosses to be about the same, with the possible exception that the value for the cross $P_1 \times P_2$ may be expected to be slightly lower than the other two due to a marked degree of dominance for resistance which is shown by the F_1 mean being the same as the resistant parent. With WARNER's (1952) formula where 1/2D was calculated as $2V_{F_2} - V_{B_1} + V_{B_2}$ the value for the cross $P_1 \times P_4$ is unusually high. This high value was caused by a high V_{F_2} with $V_{B_1} + V_{B_2}$ being about the same as for the other crosses. If only the data from replication one for the cross $P_1 \times P_4$ are used, where $V_{B_1} + V_{B_2}$ is larger, a value of 0.47 is obtained. Where D was calculated as shown by MATHER and where the obtained F_2 variances were used as the denominator, the values are as expected. Where the calculated F_2 variance was used, the values also were about as expected but with larger discrepancies. In observing the heritability values in Table 9, it would appear that heritability for Stoneville 20 resistance is in the vicinity of 45 to 50 percent. This is high enough to suggest a satisfactory degree of accuracy for selecting individual plants by phenotype alone.

The estimated numbers of effective factors, which were calculated by several different formulas, are given in Table 10. WEBER's (1950) method of calculating the nonheritable variance was used. K_1 and K_2 estimates of the number of factors suggest that the greatest difference is between P_1 and P_4, the next largest being between P_1 and P_2, with the smallest difference being between P_1 and P_3. The results obtained with the CASTLE-WRIGHT and BURTON's formulas suggest the same thing. K_2 is larger than K_1 for the crosses $P_1 \times P_2$ and $P_1 \times P_3$, but for the cross $P_1 \times P_4$, K_1 is the same as K_2. This suggests incomplete concentrations of plus genes in one parent and the presence of minus genes in the other parents for the crosses $P_1 \times P_2$ and $P_1 \times P_4$. The 1952 data calculated with the CASTLE-WRIGHT F_2 formula give essentially the same values as K_1. With BURTON's formula, with the exception of the cross $P_1 \times P_3$, the estimates are higher than those obtained with the other formula. The values calculated with the CASTLE-WRIGHT backcross formula are more irregular and do not agree very well with the values which were calculated with the F_2 formula.

DISCUSSION

The variations obtained in 1952 for the three crosses were different from those in 1951. This difference can be attributed to several things. The first was the presence of a different race of *X. malvacearum* in 1952. Isolates T_1, T_4 and T_{12} which were used in 1951 represent race 1 and another isolate, G_4, which was used in 1952 represents race 2. (This nomenclature was established by the Bacterial Blight Committee of the Cotton Disease Council at its February, 1955 meeting.)

TABLE 10

Estimated number of effective factors calculated by several formulas for the three crosses $P_1 \times P_2$, $P_1 \times P_3$ and $P_1 \times P_4$

Formula		Year of data	Cross $P_1 \times P_2$	Cross $P_1 \times P_3$	Cross $P_1 \times P_4$
$K_1 = \left(\frac{\overline{P}_1 - \overline{P}_2}{2}\right)^2 \frac{}{D}$*					
		1952	0.508	0.083	1.618
$K_2 = \frac{(1/4D+1/8H)^2}{V_{VF_2} - \text{Corr.}}$		1952	1.483	0.881	1.635
$\frac{(\overline{P}_1 - \overline{P}_2)^2}{8(V_{F_2} - \sqrt[3]{V_{P_1} \cdot V_{P_2} \cdot V_{F_1}})}$†		1951	2.275	1.311	2.419
		1952	0.489	0.086	1.479
$\frac{.25(.75-h+h^2)\ (\overline{P}_1 - \overline{P}_2)^2}{V_{B_2} - \sqrt[3]{V_{P_1} \cdot V_{P_2} \cdot V_{F_1}}}$‡		1951	2.513	1.309	2.912
		1952	0.755	0.087	2.2954
From backcross data†					
$\frac{(\overline{P}_1 - \overline{P}_2)^2}{16(V_B - \sqrt[3]{V_{P_1} \cdot V_{P_2} \cdot V_{F_1}})}$	B_1	1952	0.284	0.038	0.773
	B_2	1952	1.511	0.137	—5.079

* After MATHER (1949).
† CASTLE-WRIGHT (1921) as adapted by WEBER (1950).
‡ After BURTON (1951).

Isolates T_{12} and G_4 were used in equal amounts in 1952; thus, the 1952 results give genetic data obtained in the presence of races 1 and 2.

The effect of the two races was determined in the 1954 study where the isolates were compared. Resistance was lower in the presence of race 2 or race 2 and race 1 together than it was in the presence of race 1 alone. The combined results are essentially the same as race 2 because race 2 overshadowed race 1 and the disease grades caused by race 2 represent most plants. With the F_2 population there was an interaction between individual plants and isolates. Race 1 caused higher disease grades than race 2 on some plants while on others race 2 caused the higher disease grade. Consequently, each isolate had about the same mean disease grade. With the combined results there were more plants with higher disease grades than there were with either isolate alone. For this reason the combined results for the F_2 population had a higher mean disease grade than either isolate alone. It was also learned in other studies that the Stoneville 2B and Deltapine strains have about the same level of tolerance to race 2. As pointed out earlier, Deltapine has higher tolerance to race 1 than does Stoneville 2B.

Another factor causing the difference between 1951 and 1952 was soil moisture. The inoculation and grading were done during the month of June in both years. The 1951 planting received 1.5 inches of effective rain during the month of June while the 1952 planting received only one inch of effective rain. BIRD (1954)

reported that lower soil moisture would cause resistant plants to become less resistant while at the same time susceptible plants would become tolerant. Since the grading system is based primarily on lesion size, the higher grades were absent.

The fact has been emphasized in previous studies that Stoneville 20 resistance largely depends on favorable minor genes accompanying the major gene. Then, using KNIGHT's (1953) B_7 designation, Stoneville 20 should have the genotype $B_7B_7B_{Sm}B_{Sm}$ where B_{Sm} represents the minor gene component for Stoneville. The Deltapine parent (P_2) has a high level of tolerance which is attributed to minor genes. If it is assumed that the Deltapine minor gene component is different from the Stoneville component, the Deltapine parent would have the genotype $b_7b_7b_{Sm}b_{Sm}B_{Dm}B_{Dm}$. By the same reasoning it may be assumed that the Stoneville 2B parent (P_3) has the genotype $b_7b_7B_{Sm}{}'B_{Sm}{}'b_{Dm}b_{Dm}$ and the Acala parent (P_4) the genotype $b_7b_7b_{Sm}b_{Sm}b_{Dm}b_{Dm}$. Under these assumptions the four parents have the following genotypes:

P_1	$B_7B_7B_{Sm}B_{Sm}b_{Dm}b_{Dm}$
P_2	$b_7b_7b_{Sm}b_{Sm}B_{Dm}B_{Dm}$
P_3	$b_7b_7B_{Sm}{}'B_{Sm}{}'b_{Dm}b_{Dm}$
P_4	$b_7b_7b_{Sm}b_{Sm}b_{Dm}b_{Dm}$

The Stoneville 20 and Stoneville 2B minor gene components probably are not identical. Also, the B_{Sm} and B_{Dm} components probably are not completely different. The nonsignificant H values and the estimates of heritability point out that the dominance is not present. As shown by the significant E_1 value for the cross $P_1 \times P_4$, the nonheritable within plot variation is measurable when the minor gene level is low. The only genetic variation which is real is that which is fixable or additive. With this in mind, and by accepting the hyothesis that the additive effect of one major (B_7b_7) gene is greater and influenced less by environment in the presence of two $B__{}_m$ components but has less effect and is influenced more by environment in the present of one $B__{}_m$ or no $B__{}_m$ components, the F_2 variation which was obtained in 1951 for the three crosses can be explained.

The assumed genotypes of the four parents will also account for the F_2 variation which was obtained in 1952 when the influence of the two races of *X. malvacearum* and the 1952 environment, which were discussed above, are taken into consideration. The combined influence of the two races and the environment caused the minor genes to be almost as important as the major gene.

In studying the standard deviations of the four parents and the three F_1's which are given in Table 2, it appears that the P_1 was not homozygous resistant. This is suggested by the fact that the standard deviation for the P_1 is higher than that for P_2, P_3 and P_4 and by the standard deviations of the three F_1's being between those of the P_1 and their respective susceptible parents. The P_1 seed lot was from an open pollinated increase and it was known that about five percent outcrossing had occurred. The hybrids from outcrossing could be detected by making a pre-thinning inoculation. This was done in all P_1 plantings which were made in connection with the study and the outcross hybrids were removed during the thinning operation. For this reason the P_1 should have been homozygous for the

major resistant gene. Therefore, the apparent segregation in the P_1 was probably in the minor gene component.

The test for adequacy of scale with respect to genetic interaction showed that the scale was adequate. However, since the "C" value for the cross $P_1 \times P_4$ was significant the adequacy of the scale is probably marginal. The scaling test with respect to independence of nonheritable variation from the genotype suggests that the scale is not adequate. The results of this test are due to the variances of the P_1 and F_1's being significantly larger than the variances of the P_2, P_3 and P_4. As pointed out above, the larger P_1 and F_1 variances can be attributed to the P_1 not being homozygous in its minor gene component. If this is true the test is not valid and cannot be considered to give evidence of interaction between genotype and environment. As a whole, the tests indicate that the scale is marginally adequate, and it is doubtful that anything could be gained by changing these data to another scale.

In studying the components it is obvious that the only real heritable variance is that portion which is additive or fixable. None of the variance can be attributed to dominance. There also appears to be a real nonheritable variance which is attributed to individual plants.

The nonsignificant H values are attributed to the absence of dominance. Since dominance was not present it must be assumed that the departure of the F_1, F_2 and F_3 means from the mid-parent mean was due to chance variation. In comparing the F_1, F_2 and F_3 means of the three crosses with their respective mid-parent values, it can be seen that this is probably true.

With reference to the F_2 distribution pattern for the cross $P_1 \times P_4$ which suggested considerable dominance in 1951 and slight dominance in 1952, it may be noted that the estimate of the unfixable (H) or dominance component was not significant, and in addition, it was smaller than the estimates for the other two crosses. Less minor genes were present in the $P_1 \times P_4$ cross to influence any dominance that might be expressed by the major gene B_7, therefore, if any dominance was present the H value should have been higher or possibly significant for this cross. The absence of a significant component for dominance in the cross $P_1 \times P_4$ further supports the assumptions of the gene action of B_7 and the minor genes which were made above.

The comparison of the arithmetic and geometric means with the obtained means gave no definite information as to the gene action involved in Stoneville 20 resistance. However, the closeness of the obtained and theoretical means implies a lack of dominance and possibly a mixture of additive and multiplicative gene action conditioning Stoneville 20 resistance to bacterial blight.

MATHER's K_2 estimate of the number of effective factors is expected to be higher than the K_1 estimate, if each parent has both plus and minus allelomorphs. Therefore, the results suggest that P_2 and P_3 have plus allelomorphs and that P_4 does not. These results indicate that the assumed genotypes of the four parents, which were given above, may be correct. If linkage is present, K_1 and K_2 should be considerably smaller when linkage is not allowed for, than they are when

linkage is assumed. The K values are not very different for no linkage and linkage. Therefore, these results suggest that linkage may not be present. CASTLE's and WRIGHT's F_2 formula gave larger estimates of the number of effective factors in 1951 than in 1952. The smaller 1952 estimates were due to the smaller parental differences for that year. The backcross formula gave smaller estimates for B_1 than it did for B_2. This was because the B_1 variances were larger than the B_2 variances. The negative B_2 value for the cross $P_1 \times P_4$ was the result of an unusually small B_2 variance in replication two, as pointed out previously.

The results suggest that one effective factor is present in the cross $P_1 \times P_3$ and that at least two effective factors are present in the crosses $P_1 \times P_2$ and $P_1 \times P_4$. The major gene for resistance would be one factor and a component of minor genes would be the other. The B_{Sm} and B_{Dm} components, which were assumed above, probably have several duplicated genes so that when together the equivalent of only one component is segregating. This would be the case in the cross $P_1 \times P_2$, where under the above assumption three effective factors would be expected to be present but only two are indicated. Therefore, the estimates of the number of effective factors strongly indicate that the genotypes of the four parents are similar to those which were assumed.

MATHER (1949) pointed out that the value $\sqrt{K_2 D}$ indicates the immediate limit in which selection could cause a departure from the mid-parent value. These values for the three crosses $P_1 \times P_2$, $P_1 \times P_3$ and $P_1 \times P_4$, where the D and K_2 values calculated with all variances are respectively 1.47, 1.14 and 1.76. The departures of the P_1 from the mid-parent value for the crosses $P_1 \times P_2$, $P_1 \times P_3$ and $P_1 \times P_4$ are respectively 0.86, 0.35 and 1.75. This suggests that immediate progress could be made in selecting for higher resistance in the crosses $P_1 \times P_2$ and $P_1 \times P_3$ but no progress could be made when selecting within the cross $P_1 \times P_4$. The fact that the greatest progress can be made within the crosses $P_1 \times P_2$ and $P_1 \times P_3$ also suggests that P_1 has some minus allelomorphs for resistance and that P_2 and P_3 have some plus allelomorphs. It also suggests that P_4 has a very low level, if any, of plus genes for resistance.

Earlier genetic studies of the inheritance of Stoneville 20 resistance, as pointed out previously, were not in agreement as to whether resistance was recessive or dominant. The 1952 data show how such conflicting data could be obtained when only the F_1 and F_2 generations are studied in a single genetic background. The cross $P_1 \times P_2$ suggests that resistance is dominant, the cross $P_1 \times P_3$ suggests no dominance and the cross $P_1 \times P_4$ suggests that resistance is recessive. Yet when these data are analyzed statistically there is no evidence of dominance. The 1952 data emphasizes the importance of the minor gene component in resistance, as did the earlier genetic studies. However, the 1952 data show how the additive effect of the effective factors can give F_2 distribution curves which can be misinterpreted as suggesting dominance.

The statistical analysis of Stoneville 20 resistance gave information which clarified earlier genetic studies. At the same time, additional information was obtained which led to a plausible prediction of the genotypes of the four parents

as well as the gene action concerned. This certainly suggests that statistical genetic studies can be made with accuracy where the phenotype is measured by assigning disease grades.

The 1952 genetic data were collected under conditions unfavorable for Stoneville 20 resistance, namely the presence of *X. malvacearum* race 2 which is more virulent than race 1 for Stoneville 20 resistance and environmental conditions which interfered with the physiological genetic system for resistance. The above two factors are current problems confronting plant breeders who are developing bacterial blight resistant strains of cotton. These data give important information which can be utilized in solving these two problems.

The knowledge of the physiological genetic system of resistance and how it is influenced by environment should be extremely useful. It indicates that the breeder when selecting for resistance should consider the level and the variability of the soil moisture and the available soil nitrogen within the breeding nursery. If these two factors are unfavorable or variable he should not be too critical, or many lines may be discarded which have an acceptable genetic level of resistance. This would unnecessarily reduce the number of lines available for agronomic selection. At the same time, if these two factors are favorable and have a low variation level, the breeder should be extremely critical when selecting for resistance. These facts alone would greatly increase the efficiency of a bacterial blight breeding program.

The results of this study strongly suggest that similar statistical genetic studies, where several genetic backgrounds are used, made with other *G. hirsutum* L. characters should provide valuable information as to the roles of major and minor genes in conditioning a phenotypic expression.

SUMMARY

Four strains of *Gossypium hirsutum* L. which were selected because of their differential response to infection by *Xanthomonas malvacearum* were used in statistical studies of the inheritance of Stoneville 20 resistance to the bacterial blight disease of cotton.

X. malvacearum races 1 and 2 were used as a composite inoculum for inoculating the individual plants used in the genetic investigations. Separate studies, which were made with both races, showed that Stoneville 20 was highly resistant to race 1 but it was only slightly resistant to race 2. However, in an F_2 population from the cross Stoneville 20 × Stoneville 2B some plants were resistant to race 1 but susceptible to race 2 and some were resistant to race 2 but susceptible to race 1. A small proportion of the F_2 plants were resistant to both races.

The genetic data show how a study of Stoneville 20 resistance which is made within a single genetic background and with only F_1 and F_2 generations could be misleading as to whether dominance was present or absent and as to whether resistance was dominant or recessive.

The total phenotypic variance was partitioned into its fixable genetic, unfixable genetic and nonheritable components. The only component of the variance which

was highly significant was the one due to the fixable or additive genetic variation. The compound due to nonheritable genetic variation of individual plants was slightly significant. No measurable unfixable component or variation due to dominance was present.

The data suggest that the factors conditioning Stoneville 20 resistance are not linked. A measurable amount of genetic interaction was present.

Several formulas for estimating the number of effective factors were compared. The results indicate that two effective factors condition Stoneville 20 resistance. One is the major gene B_7 and the other is a component of minor genes B_{Sm}. Deltapine and Stoneville 2B each have one effective factor and Acala has no effective factors. The effective factors in Deltapine and Stoneville 2B are attributed to minor gene components, B_{Dm} and B_{Sm}, respectively, which as a whole are different but they have some genes in common so that when together they appear as one effective factor.

The results support the hypothesis that the four strains have the following genotypes:

Stoneville 20	$B_7B_7B_{Sm}B_{Sm}b_{Dm}b_{Dm}$
Deltapine	$b_7b_7b_{Sm}b_{Sm}B_{Dm}B_{Dm}$
Stoneville 2B	$b_7b_7B_{Sm}{}'B_{Sm}{}'b_{Dm}b_{Dm}$
Acala	$b_7b_7b_{Sm}b_{Sm}b_{Dm}b_{Dm}$

The major and minor genes are additive with no dominance. The additive effect of one major (B_7b_7) gene is greater and influenced less by environment in the presence of two $B__{}_m$ components but has less effect and is influenced more by environment in the presence of one $B__{}_m$ component.

Several methods of estimating heritability of differences among F_2 plants were compared. The results suggest a value of about 45–50 percent as an estimate of heritability for Stoneville 20 resistance.

The genetic data strongly indicate that if genetic variability is maintained, material with the Stoneville 20 factors which resist *X. malvacearum* races 1 and 2 can be obtained by selecting individual plants for resistance. The data indicate that progress would be better and higher resistance could be obtained within material with two or more components of minor genes.

The results obtained with this study show that statistical genetic studies of characters which are measured phenotypically by assigning disease grades can be successful.

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